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Clinical and Experimental

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A COMPARATIVE STUDY OF THE CLINICAL AND METABOLIC EFFECTS OF ACTH DERIVED FROM HOGS, SHEEP, AND CATTLE

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THE earliest commercial preparations of adrenocorticotropic hormone (ACTH) were derived from hog pituitaries. To meet an increasing demand for ACTH, attempts have been made to utilize the pituitaries of various other mammalian species, as well as to develop new processes for increasing the yield of active material. As new preparations become available, the question arises as to whether qualitative or quantitative differences exist in their biological activities. There is no a priori assurance that ACTH derived from different species will have identical biological effects. Furthermore, it is possible that different processes of extracting ACTH from raw material may yield products which differ in biological activity. For example, there have already been reports which indicate that adrenal ascorbic-acid-depleting activity may not parallel adrenal weight-maintaining activity.[1] The question about which the present study developed was whether the clinical and metabolic effects of ACTH would vary depending upon the species from which the pituitaries were obtained or the method of preparation.

Five different preparations of ACTH were compared.* The material utilized included lyophilized ACTH prepared by the Armour Company from the pituitary glands of hogs, sheep, and cattle, ACTH prepared from hog pituitaries by the National Drug Company, and ACTH prepared from sheep pituitaries by Dr. C. H. Li.** Four patients suffering from rheumatoid arthritis served as subjects for this investigation.

METHODS

The subjects were maintained on a metabolic balance regimen, and clinical and laboratory indices of ACTH activity, as detailed below, were followed during treatment periods of six to twelve days and appropriate control periods. During treatment periods each patient received 100 International Units of ACTH daily by intramuscular injection. Throughout the study each patient received 0.9 Gm. of acetylsalicylic acid five times daily. The physical activities of each patient were practically constant from day to day. For any one patient, the total calories, fat, carbohydrate, protein, sodium, potassium, calcium, and phosphorus of the diet were constant from day to day. At frequent intervals a duplicate of an entire day's

From the Metabolic Unit for Research in Arthritis and Related Diseases and the Divisions of Medicine and Physiology, University of California School of Medicine, San Francisco and Berkeley.

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*The authors are indebted to Dr. H. Cluxton of the Armour Laboratories, to Dr. G. J. Martin of the National Drug Company, and to Dr. C. H. Li for generous supplies of ACTH.

**Prepared by a method described elsewhere.[2]

diet was homogenized, and aliquots were analyzed for nitrogen, sodium, potassium, calcium, and phosphorus. Because several analyses gave reproducible results which compared closely with values calculated from tables, it was believed unnecessary to analyze each day's diet.

Twenty-four hour collections of urine were preserved with hydrochloric acid. Carmine was used to delimit stool-collection periods, which were six days in length. In preparation for mineral analysis, dietary and stool aliquots were dry-ashed at 400° C. for four hours. On alternate days blood was drawn under fasting conditions for chemical analyses and counts of circulating eosinophiles.

The following laboratory methods and determinations were employed:

Sodium and potassium: A Perkin-Elmer flame photometer (Model 52A) with lithium as an internal standard was used.

Phosphorus: Method of Fiske and SubbaRow.[3]

Calcium: Method of Michaels and associates.[4]

Nitrogen: A steam-distillation micro-Kjeldahl method.

Urinary 17-ketosteroids: Method of Hawk, Oser, and Summerson.[5]

Blood glucose: Method of Nelson.[6]

Total circulating eosinophiles: Method recommended by Madison;[7] Levy counting chamber.

Clinical examinations were carried out daily by two independent observers. In evaluating the response to therapy, greater stress was placed on objective changes in the activity of the disease process (judged by fever, synovial thickening, accumulation of synovial fluid, local tenderness, and limitation of motion) than on symptomatic improvement. Actually, subjective changes paralleled objective changes rather closely.

CLINICAL MATERIAL

CASE 1.—A. A. (UCH 181489), a 21-year-old professional baseball player, had moderately severe generalized rheumatoid arthritis of two years' duration. This patient was observed according to the following schedule:

Equilibration	6 days
Control	12 days
Li sheep ACTH, 25 units i.m. every 6 hours	6 days
Control	12 days
Armour hog ACTH, 25 units i.m. every 6 hours	6 days
Control	12 days
Armour bovine ACTH, 25 units i.m. every 6 hours	6 days
Control	12 days
Armour sheep ACTH, 25 units i.m. every 6 hours	6 days
Control	12 days
National Drug Company hog ACTH, 25 units i.m. every 6 hours	6 days
Control	3 days

CASE 2.—MRS. L. G. (UCH 121954), a 33-year-old singer, had severe generalized rheumatoid arthritis of five years' duration. Previous treatment for arthritis had consisted of several courses of chrysotherapy; she had received no ACTH or cortisone. Three years previously she had had an hysterectomy and bilateral ovariectomy for endometriosis. Subsequent replacement therapy with estrogens had been discontinued one month before the present study. This patient was observed according to the following schedule:

Equilibration	6 days
Control	6 days
Potassium acetate,* 12 Gm. three times daily by mouth	6 days
Armour hog ACTH, 25 units i.m. every 6 hours, plus potassium acetate as above	6 days
Control	18 days
Li sheep ACTH, 25 units i.m. every 6 hours	6 days
Control	6 days

*Except for its influence upon sodium and potassium metabolism, the potassium acetate administered to two of these patients interfered in no way with the effects of ACTH observed in this study. The effects of potassium upon electrolyte metabolism in these patients will be reported elsewhere.

Study suspended for 5 weeks	
Equilibration	24 days
Control	6 days
National Drug Company hog ACTH, 25 units i.m. every 6 hours	6 days
Control	12 days

CASE 3.—Mrs. R. D. (UCH 187886), a 48-year-old cannery worker, suffered from mild generalized rheumatoid arthritis of one year's duration. She had received no previous treatment for arthritis. This patient was studied according to the following schedule:

Equilibration	6 days
Control	6 days
Armour hog ACTH, 25 units i.m. every 6 hours	6 days
Armour hog ACTH, as above, plus potassium acetate 12 Gm. three times daily by mouth	6 days
Potassium acetate, as above	6 days
Control	12 days
Li sheep ACTH, 25 units i.m. every 6 hours	6 days
Control	6 days

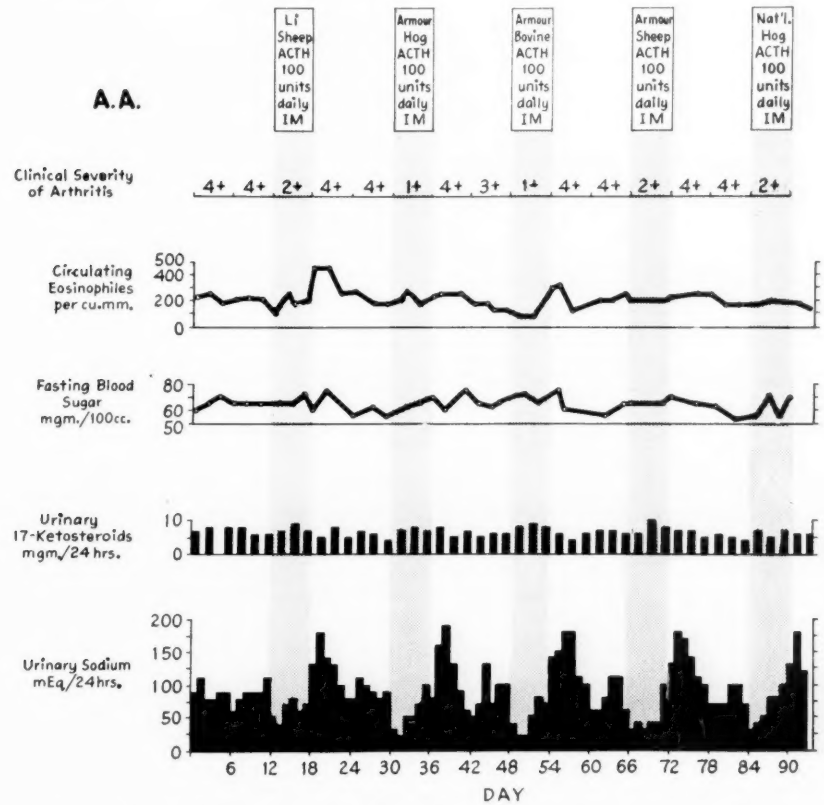

Fig. 1.—(Patient A. A.) Influence of five ACTH preparations upon arthritic activity, circulating eosinophiles, fasting blood sugar, urinary 17-ketosteroids, and urinary sodium.

CASE 4.—J. K. (UCH 175983), a 45-year-old miner, suffered from severely crippling generalized rheumatoid arthritis of three years' duration. In addition, he exhibited marked constitutional manifestations of the disease, notably fever, anorexia, lassitude, and general debility. The clinical response of this patient to ACTH and to cortisone was remarkably good, but whenever the hormones were withdrawn, the ensuing exacerbation (characterized by fever, pain, prostration, tachycardia, and hypotension) was so severe that practically continuous

treatment was necessary. For four months prior to the present study, therefore, he had received from 60 to 100 units of ACTH daily. Observations on this patient were made according to the following schedule:

Dietary equilibration (while receiving ACTH, 60 units daily)	6 days
Armour hog ACTH, 25 units i.m. every 6 hours	6 days
Li sheep ACTH, 25 units i.m. every 6 hours	12 days
Armour hog ACTH, as above	6 days
"Control" withdrawal of ACTH	1 day
Li sheep ACTH, as above	5 days

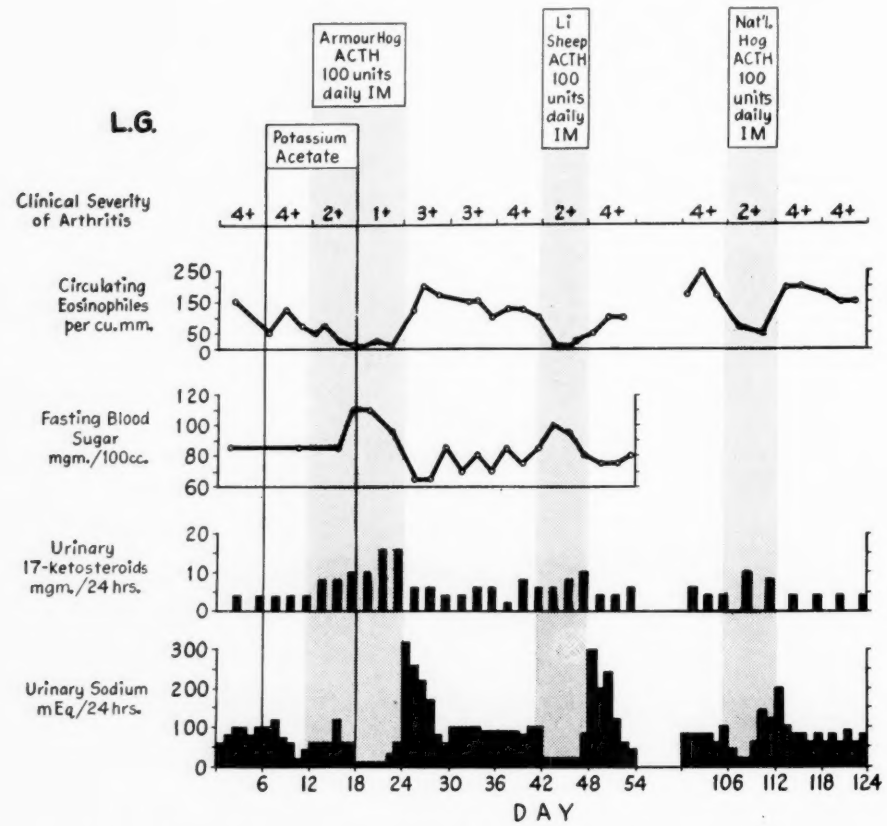

Fig. 2.—(Patient L. G.) Influence of three ACTH preparations upon arthritic activity, circulating eosinophiles, fasting blood sugar, urinary 17-ketosteroids, and urinary sodium.

RESULTS

Clinical Observations.—All four patients demonstrated an unequivocal therapeutic response to the administration of ACTH, regardless of the preparation used. *Subjective* responses included diminution of pain and stiffness of the joints, increased desire for activity, and an increased feeling of well-being. *Objective* responses to each type of ACTH included diminution of synovial thickening, decrease in the quantity of synovial fluid, increased range of painless motion of the joints, and disappearance of fever whenever it was present. The clinical improvement became apparent within twenty-four hours after treat-

ment was started and lasted as long as treatment was continued. Exacerbations of arthritic activity began within twenty-four hours of the time treatment was withdrawn. The therapeutic efficacy of the different ACTH preparations was roughly equivalent.

In patient J. K., even a minor reduction in the amount of ACTH (for example, from a dose of 80 units daily to a dose of 60 units daily) was accompanied by exacerbation of the disease. Yet a change from Armour hog ACTH to the same dose (in terms of International Units) of Li sheep ACTH was not accompanied by any significant clinical or metabolic change (Fig. 4). The severe exacerbation which occurred whenever ACTH was withdrawn was controlled equally well by either Li sheep ACTH or Armour hog ACTH.

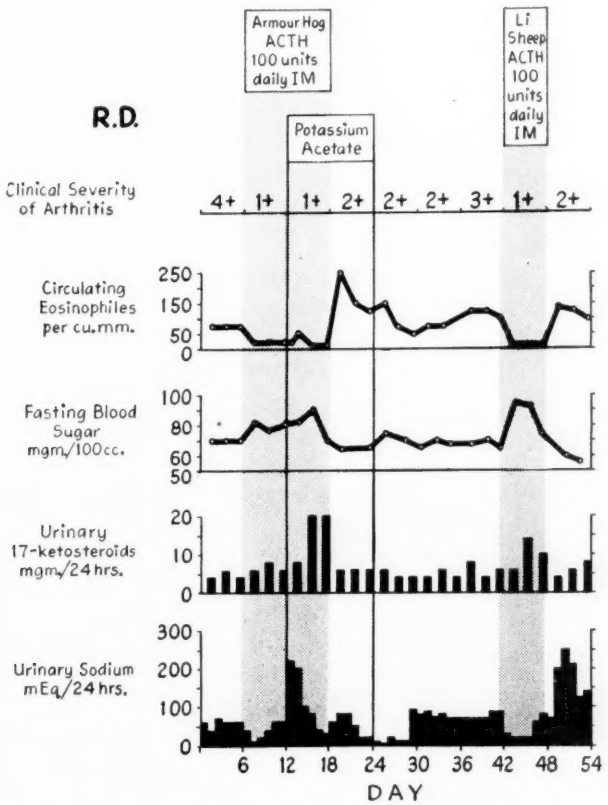

Fig. 3.—(Patient R. D.) Influence of two ACTH preparations upon arthritic activity, circulating eosinophiles, fasting blood sugar, urinary 17-ketosteroids, and urinary sodium.

Metabolic Observations.—The metabolic responses to all the preparations were essentially similar. Representative data are presented in Figs. 1, 2, 3, and 4. Administration of each preparation led to an initial phase of sodium retention, and withdrawal was followed promptly by sodium loss. Patient J. K. was already receiving ACTH when this study was started, therefore it was not possible to observe the initial phase of sodium retention in this patient. How-

ever, withdrawal of ACTH for one day resulted in excretion of 300 milliequivalents of sodium in one twenty-four hour period (Fig. 4, day 25). Reinstitution of ACTH therapy was then followed by a period of sodium retention. In all patients, retention of sodium was accompanied by retention of water, as evidenced by a moderate increment in body weight; diuresis of sodium was accompanied by a loss in weight. During the first twenty-four hour period following the withdrawal of each ACTH preparation, urinary excretion of potassium decreased; this effect did not persist beyond twenty-four hours. When computed on the basis of six-day periods, potassium balance was not appreciably affected by any of the ACTH preparations in the doses employed. Serum potassium levels fell consistently during administration of ACTH. In one patient (J. K.), who had received large doses of ACTH for several months prior to this study, the serum potassium levels were very low (approximately 2 to 3 milliequivalents per liter) and tended to decrease further during the study.

Comparable increases in urinary excretion of 17-ketosteroids were found with each type of ACTH.

Frequent determinations of fasting blood sugar levels suggested that the various types of ACTH did not differ in their effect upon carbohydrate metabolism. Patient J. K. developed fasting blood sugar levels within the "diabetic" range (110 to 130 mg. per 100 ml.); this hyperglycemia was accompanied by mild glycosuria and was equally notable with either the Li sheep ACTH or the Armour hog ACTH. L. G. and R. D. displayed transient increases in fasting blood sugar values while receiving the Li sheep and the Armour hog preparations of ACTH. A. A. showed no significant change in fasting blood sugar levels with any type of ACTH.

Eosinopenia occurred in three of our four subjects whenever they received ACTH of any type. One subject (A. A.), whose response to ACTH (judging by all laboratory criteria) was less than that of the other subjects, failed to show a convincing decrease in eosinophiles in response to any of the five preparations given intramuscularly. Incidentally, the apparent refractoriness of the eosinophiles of this patient had been noted prior to this study, when he had first

TABLE I. EOSINOPENIC EFFECTS OF VARIOUS PREPARATIONS OF ACTH WHEN ADMINISTERED INTRAMUSCULARLY OR INTRAVENOUSLY
PATIENT A. A.

TREATMENT	PER CENT CHANGE IN EOSINOPHILES
No ACTH 8-hour infusion of dextrose in water, i.v.	7% increase at termination of infusion
Armour lyophilized hog ACTH 50 units, i.m.	15% decrease 4 hours after injection
Li sheep ACTH 50 units, i.m.	28% decrease 4 hours after injection
Li sheep ACTH 2 units, i.v., 8-hour infusion	91% decrease at termination of infusion
Purified ACTH 25 units, i.m., every 6 hours for one day	90% decrease 6 hours after last injection
Armour lyophilized hog ACTH 25 units, i.m., every 6 hours for one day	20% decrease 6 hours after last injection

received ACTH during a Thorn test. It is believed, therefore, that refractoriness in this instance was not induced in the course of ACTH therapy. Further studies, therefore, were carried out to clarify the nature of the refractoriness of this subject (see Table I). Fifty units of either Li sheep ACTH or Armour hog ACTH injected intramuscularly failed to induce a significant decrease in eosinophiles. On the other hand, when as little as two units of the Li sheep

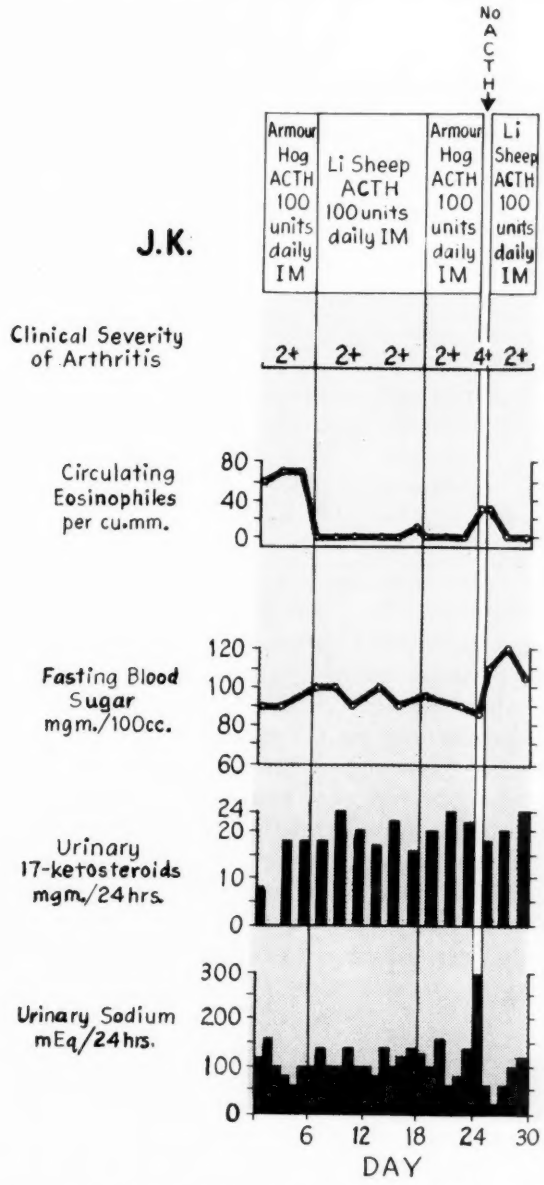

Fig. 4.—(Patient J. K.) Influence of two ACTH preparations upon arthritic activity, circulating eosinophiles, fasting blood sugar, urinary 17-ketosteroids, and urinary sodium.

preparation were infused intravenously over an eight-hour period, marked eosinopenia resulted. Twenty-five units of ACTH* purified by a modification of the method of Payne and associates,[8] given intramuscularly every six hours for one day, was accompanied by a 90 per cent decrease in eosinophiles and marked clinical improvement of the patient's arthritis. When Armour hog ACTH was then substituted in the same dosage, the clinical improvement was fairly well maintained, despite an increase in eosinophiles toward pre-treatment levels.

DISCUSSION

No qualitative differences in either antirheumatic or metabolic effects were found among the various preparations used in this study, however, minor quantitative differences did appear. These were not particularly striking, in view of the variability inherent in this type of study and the fact that the standardization of the dosage of ACTH itself is based upon a biological assay which has a 20 per cent variability.[9]

Even though our four subjects were given the same dosage of ACTH (100 units daily), the responsiveness to ACTH differed from patient to patient. This difference was consistent in that the response of patient A. A. was always less marked than that of the other subjects, regardless of the type of ACTH used. The normal eosinopenic response of this subject to small doses of ACTH injected intravenously suggests that his diminished responsiveness to ACTH given intramuscularly was due to increased inactivation of ACTH at the site of injection. If so, the increased inactivation is apparently favored by some material in the commercial preparations which was not present in the more highly purified preparation.

Ordinarily the eosinopenic response is one of the most sensitive indices of increased adrenocortical activity. The fact that patient A. A. regularly displayed clinical improvement and retention of sodium in response to ACTH without displaying eosinopenia could be explained by an eosinophilic reaction to foreign material injected with the ACTH.

It has been reported that certain patients may become refractory to repeated intramuscularly injections of the less highly purified preparations of ACTH.[10] It appears, however, that patient A. A. may be representative of another group of individuals who exhibit a subnormal response to intramuscular injections of ACTH spontaneously, without having been sensitized by previous injections. Furthermore, since this patient exhibited comparable refractoriness to ACTH derived from hogs, sheep, and cattle, it may be inferred that there is a type of refractoriness which is not specific for any one species-source of ACTH.

SUMMARY

Metabolic and clinical observations were made on four patients with rheumatoid arthritis treated with ACTH derived by various processes from the pituitaries of hogs, sheep, and cattle.

ACTH from each source was effective in inducing clinical remissions of arthritic activity.

*The purified ACTH was kindly supplied by Parke, Davis & Company, Detroit, Mich.

ACTH from each source induced sodium retention, a fall in serum potassium, a rise in fasting blood sugar, a rise in urinary excretion of 17-ketosteroids, and a decrease in circulating eosinophiles.

No significant differences were noted in the qualitative or quantitative effects of the various types of ACTH when they were administered in doses which were similar according to standard biological assay methods.

One subject exhibited partial refractoriness to intramuscular injections of ACTH. This refractoriness was not evident when ACTH was administered intravenously or when purified ACTH was administered intramuscularly. In this instance the refractoriness was not induced by previous therapy and it was not specific for any species-source of ACTH.

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ELECTROPHORETIC STUDIES ON RHEUMATOID ARTHRITIS PATIENTS TREATED WITH CORTISONE

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PRIMARILY because of an interest in plasma mucoproteins, changes in electrophoresis patterns of the sera of five patients being treated with cortisone were carried out several years ago. The studies were not completed in time to be included in the original report which Boland and Headley[1] made on their patients, and were not published later because of the negative nature of the results. However, several papers have now appeared[2-5] in which conflicting results have been obtained. It seems desirable to report our results briefly, which show that cortisone has no immediate effect on the serum proteins which is detectable by electrophoresis.

EXPERIMENTAL

The patients were M. L., J. S., L. D., A. H., and M. S., of a group already reported upon.[1] Cortisone was injected (300 mg.) on day 1, and for the next seven days (100 mg.), except in the case of L. D., who received 200 mg. on each of the first two days. Cholesterol injections were given before the cortisone period, and during a seven-day period following cortisone.

The total protein in the serum samples was determined by a biuret method,[6] and the albumin, α_1-, α_2-, β-, and γ-globulins were calculated from the total protein and relative areas of the electrophoretic components at pH 8.5. The amounts of components M-1 and M-2 were determined in the same way, from electrophoresis patterns at pH 4.5[7]

TABLE I. SERUM PROTEIN CONCENTRATIONS IN RHEUMATOID ARTHRITIS, AS INFLUENCED BY CORTISONE. CORTISONE ADMINISTERED ON DAYS 1 THROUGH 8.

CASE	DAY	PROTEIN, GM. PER 100 ML. SERUM							
		TOTAL	ALB.	α_1	α_2	β	α	M-1	M-2
M. L.	0	6.2	3.0	0.4	0.9	1.2	0.7	0.05	0.4
	5	5.9	--	--	--	--	--	0.05	0.5
	8	6.0	3.0	0.4	0.8	1.1	0.7	0.10	0.5
	15	6.0	3.0	0.3	1.4	0.6	0.7	0.10	0.6
J. S.	0	5.5	1.6	0.8	1.0	0.9	1.2	0.25	0.8
	5	6.1	2.0	0.6	0.9	1.3	1.3	0.15	0.7
	8	5.1	1.8	0.6	0.7	1.0	1.0	0.10	0.5
	15	5.1	2.0	0.6	0.6	1.0	0.9	0.15	0.6
	42	6.8	2.9	0.5	0.6	1.0	1.8	0.10	0.3
L. D.	0	6.0	2.7	0.3	0.5	1.3	1.2	0.05	0.3
	8	6.5	--	--	--	--	--	0.10	0.3
	15	6.2	2.8	0.3	0.8	1.4	1.0	0.10	0.4
A. H.	0	5.8	2.5	0.5	0.7	0.8	1.2	0.1	0.4
	8	5.0	2.1	0.3	0.9	0.7	1.0	0.1	0.3
M. S.	0	6.2	2.9	0.4	0.7	1.1	1.1	0.10	0.4
	8	6.5	2.9	0.4	0.7	1.5	1.0	0.05	0.5
	15	6.4	3.0	0.4	0.7	1.5	0.7	0.05	0.4

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RESULTS

The values for the plasma proteins are presented in Table I, and it will be seen that no consistent response is evident.

DISCUSSION

The results indicate that a clinical response can be obtained without any significant effect on the serum proteins, including M-1 and M-2. J. S. was followed for a longer period because a remission was obtained, and the serum proteins showed a striking response after a considerable period, as well as the more immediate reduction of the initial high levels of M-1 and M-2.

SUMMARY

Studies of electrophoresis patterns of five patients treated with cortisone confirm reports [2, 4, 5] that the concentrations of serum proteins are not necessarily altered by treatment with cortisone.

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VITAMIN A CONCENTRATION IN THE LIVER IN THE NEPHROTIC SYNDROME

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IN RECENT studies in this laboratory,[1, 2] an abnormality was demonstrated in the metabolism of vitamin A in children with the nephrotic syndrome (nephrotic stage of glomerulonephritis).

The fasting serum vitamin A concentrations were much higher than normal (the average was seven times higher). In addition, following a test dose of vitamin A, the serum vitamin A concentrations reached much higher levels than in the normal and remained above the pretest dose level for longer periods of time.

Since by far most of the tissue vitamin A is stored in the liver,[3, 4] and since Josephs'[5] work suggests that the liver normally has limited ability to take up a large quantity of vitamin A, it was thought that in children with the nephrotic syndrome the prolonged elevation of vitamin A concentration in the serum might be due to failure by the liver to utilize or store vitamin A at even the normal rate.

Popper,[6] using fluorescence microscopy, found no change in liver vitamin A in twenty-two cases of renal disease in adults as compared to a number of other diseases. Moore,[7] using the antimony trichloride method, studied twelve cases of "subacute nephritis" from patients 15 to 59 years of age, and found the liver vitamin A "reserve" to be below the "ordinary disease level." However, it is not stated whether any of these cases presented clinical manifestations of the nephrotic syndrome.

It was, therefore, important to determine the amount of vitamin A in the livers of children who had clinical manifestations of this syndrome and to compare these concentrations with the liver vitamin A concentrations in normal subjects and in patients with other diseases.

METHODS

Liver specimens were obtained at autopsy. The type of cases is described below. The control data were taken consecutively from all the obtainable autopsy material. Samples of about 0.25 Gm. were weighed and processed immediately. They were digested with 5 ml. of 6 per cent alcoholic solution of KOH at 70° C. in a water bath.[8] When disintegration of the tissues was complete, the solution was made up to 10 ml. in a volumetric flask with 95 per cent alcohol. The vitamin A determination was done on this solution using Bessey's method.[9] The final calculation was modified slightly in order to yield the vitamin concentration in terms of μg per gram of liver.

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RESULTS

Table I shows the vitamin A concentrations in the livers of three children who died of nephritis with the nephrotic syndrome,* two (J. G. and B. B.) who died of toxic nephrosis due to poisoning, and in seventeen other patients who died from various other causes. Several determinations on various portions of the livers of the three patients with nephrotic syndrome showed that they contained from 585 μg to 2,105 μg per gram (average 1,044). The hilar portions of the livers contained more than the right or left portions. The livers of the patients with toxic nephrosis contained 353 μg and 415 μg per gram. The seventeen other cases revealed from 1.4 to 375 μg per gram (average 141).

The latter data do not represent a completely satisfactory control because they were not obtained from normal subjects. However, Moore and Cooper[10] reported that "in the livers of seventy-one civilians between the ages of 15 and 59 years who died in Britain through accident in the years 1941-1944," the liver vitamin A concentrations varied from 36.6 to 273 μg per gram (average 97.2). The livers of seven children under 15 years of age who died suddenly showed 70.5 to 339 μg per gram (average 165). Ellison and Moore[11] previously reported that in the livers of twelve children from 4 months to 14 years of age (mean age 5 7/12 years) who died within seven days of their injuries, the vitamin A concentrations varied from 9.3 to 69 μg per gram (average 39).

Contrary to our expectations, therefore, the vitamin A content of the liver in the nephrotic syndrome is very high. It is from two to forty times greater than in the other patients in this study, and the values in the three cases are six to over twenty times higher than the average of seventy-one normal individuals reported from England. The lowest value in the three cases of nephrotic syndrome was more than twice as high as the highest in the seventy-one normal individuals.

The child with the nephrotic syndrome who had the lowest liver vitamin A concentrations (585 to 613 μg per gram) had been deliberately deprived of all sources of added vitamin A in her diet for almost two years. In spite of this, her liver vitamin A was very high. Her serum vitamin A was 79 μg per cent after she had been taken off added vitamin A in her diet for about six months. However, vitamin A levels after test doses continued to be typically very high as in other cases of nephrotic syndrome.

It is interesting that one patient with toxic nephrosis due to sulfonamide had a higher liver vitamin A concentration than any of the remaining patients and that the other patient with toxic nephrosis due to salicylates also had one of the highest levels.

DISCUSSION

In a previous study[1] it was suggested that the high serum vitamin A levels observed in the nephrotic syndrome following a test dose might be due to the fact that the liver in this syndrome does not take up vitamin A as rapidly as in the normal. This study shows that the liver in the nephrotic syndrome con-

*Two of these patients had received no ACTH or cortisone (J. S. and J. K.). One had received cortisone one year before death (V. S.).

TABLE I. CONCENTRATION OF VITAMIN A IN THE LIVER

	PATIENT	AGE	SEX	PATHOLOGIC DIAGNOSES	VITAMIN A IN LIVER μG/GM.	
Nephrotic syndrome	J. S.	3 yr.	M	Glomerulonephritis, subacute Edema, generalized Hydrothorax, bilateral Hydropericardium Hydroperitoneum Hydrocele, bilateral Edema of gall bladder Interstitial edema, myocardium Cloudy swelling, heart, liver, and kidneys Interstitial pneumonitis with superimposed acute bronchopneumonia	right left hilus	1742 1682 2105
	J. K.	4 yr.	M	Glomerulonephritis, acute, superimposed upon a subacute glomerulonephritis Edema, generalized Hydroperitoneum Hydrocele, bilateral Interstitial edema, myocardium Cloudy swelling, heart, liver, and kidneys Pneumonia, primary atypical with superimposed acute bronchopneumonia	right left hilus	659 628 792
	V. S.	4 yr.	F	Glomerulonephritis, acute, superimposed upon a subacute glomerulonephritis Ascites Hydrothorax, bilateral Pulmonary edema Hypertrophy and dilatation of heart Atheromatosis of aorta and mitral valve	right left hilus	585 591 613
Other diseases	J. G.	11 mo.	F	Toxic nephrosis, acute, due to crystallization of sulfonamide drug Occlusion, left ureterovesical orifice Hydronephrosis left and hydroureter left Pneumonitis, interstitial Severe cloudy swelling of liver Hydrothorax, bilateral, moderate Ascites, slight	415	
	S. R.	58 yr.	M	Arteriosclerotic and hypertensive heart disease Nephrosclerosis Coronary artery occlusion	376	
	B. B.	4 mo.	M	Toxic nephrosis, acute, due to salicylate poisoning Ileocecitis, acute Bronchopneumonia, upper right lung Fatty metamorphosis of liver Cloudy swelling of myocardium, liver and kidneys Hydrothorax, bilateral, moderate Ascites, moderate Anasarca Passive congestion of lungs	353	

TABLE I.—CONT'D

PATIENT	AGE	SEX	PATHOLOGIC DIAGNOSES	VITAMIN A IN LIVER μG/GM.
J. G.	64 yr.	M	Arteriosclerosis, generalized Coronary sclerosis and occlusions Hyperemia of lungs, liver, and spleen Old infarcts in heart, spleen, kidney	288
M. W.	7 yr.	F	Hodgkin's sarcoma, involving liver Ascites	277
D. R.	62 yr.	M	Carcinoma of esophagus	216
D. W.	45 yr.	F	Cancer of breast with metastasis to liver, lungs, kidney, spleen, etc.	203
H. L.	63 yr.	M	Coronary arteriosclerosis Myocardial infarction	170
F. N.	13 mo.	M	Malignant lymphoma involving spleen, liver, kidney, epicardium, lymph nodes, and brain Pneumonia, interstitial	145
E. J.	53 yr.	F	Cor pulmonale Bronchiectasis, chronic Chronic passive hyperemia of liver, spleen, and kidneys	106
G. W.	44 yr.	M	Pyelonephritis, chronic with hypertension, uremia, and congestive heart failure	91
J. J.	73 yr.	M	Heart failure Myocardial fibrosis Multiple emboli	91
C. O.	15 yr.	F	Rheumatic fever Heart failure, congestive	79
M. F.	74 yr.	F	Arteriosclerosis, generalized Bronchopneumonia Nephrosclerosis, arteriolar	57
E. Z.	6 mo.	M	Septicemia probably due to meningococci Bilateral diffuse hemorrhage into adrenal glands (Waterhouse-Friderichsen syndrome) Cloudy swelling of liver and kidneys Bronchopneumonia, early, acute, confluent	35
D. K.	9 yr.	M	Poliomyelitis, acute bulbospinal Laryngotracheobronchitis, acute, with mucous plugs in bronchi and bronchioles Fatty infiltration, myocardium right ventricle Severe fatty metamorphosis of liver	19
S. H.	64 yr.	F	Arteriosclerotic heart disease Cerebral hemorrhage Pyelonephritis, chronic	12
M. S.	4 days	F	Bronchopneumonia Ileus-intestinal obstruction	11
E. C.	40 yr.	F	Leukemia, chronic myelogenous	1

tains considerably more vitamin A than normal. This could be because the liver takes it up more rapidly and/or utilizes or liberates it more slowly than is normal. Perhaps the liver in this condition, having already taken up and retained more vitamin A than is normal, and having thus become saturated, is no longer able to handle more. Whether these findings are secondary to other changes in the liver or are indicative of a metabolic disturbance of primary importance in this syndrome remains to be determined.

CONCLUSIONS

The livers of three patients with the nephrotic syndrome were found to contain very high concentrations of vitamin A as compared to the livers of other patients and to normal livers. These data support previous data[1] which showed that there is an alteration in the manner in which vitamin A is metabolized in this syndrome. Whether the abnormally high blood and liver vitamin A levels in the nephrotic syndrome are indicative of a change of pathogenetic importance must await further information.

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BLOOD PLASMA PEPSINOGEN

I. The Source, Properties, and Assay of the Proteolytic Activity of Plasma at Acid Reactions

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THE presence in the urine of an enzyme with proteolytic activity at strongly acid reactions is now well established.[1] The disappearance of this enzyme from the urine after gastrectomy,[2, 3] the decreased concentration in the urine of patients with pernicious anemia,[3, 4] the increased activity in the urine of patients with duodenal ulcer,[5, 6] and the increased concentration in the urine of dogs after the intravenous administration of pepsinogen[3] led to the hypothesis that the peptic cells of the stomach secrete pepsinogen directly into the circulation from which it is removed by the kidneys and excreted as such.[1, 7] This hypothesis received support in the more recent demonstration of a highly significant positive correlation between the secretion of pepsin directly in the gastric lumen and the excretion of pepsinogen in the urine.[8]

The thesis that urinary pepsinogen is derived from the secretion of pepsinogen by the stomach necessarily implies the transport of this enzyme by the blood. That plasma and serum display proteolytic activity at acid reactions has been demonstrated by Saxl,[9] Leoper, and Debray,[10] Gottlieb,[11] Kleinman and Schaar,[12] Kamner, Peranio, and Bruger[13] and others, but the nature of the responsible systems remains obscure. The present report deals with studies on the nature of the enzymes responsible for the proteolytic activity of plasma at acid reactions with a method for the assay of plasma pepsinogen.

METHODS

A modification of Anson's procedure for the determination of pepsin[14] was utilized for the assay of the proteolytic activity of plasma at strongly acid reactions. Two milliliters of plasma from oxalated blood* were added to 10 ml. of a 2.5 per cent hemoglobin substrate[14] and the mixture was adjusted to the desired pH with 3N HCl and then transferred quantitatively to a graduated cylinder and brought to a volume of 15 ml. with distilled water. An aliquot of 6 ml. of the plasma-substrate mixture was incubated for twenty-four hours in a water bath at 37° C. At the end of this period, the reaction was stopped by the addition of 10 ml. of 0.3N trichloracetic acid, the mixture filtered, and the concentration of the acid-soluble "tyrosine-like" substances in an appropriate amount of the filtrate was determined by the Folin-Ciocalteu method[16] adapted to the use of a spectrophotometer. The concentration of chromogenic substances originally present in the plasma-hemoglobin mixture was determined by an analysis of the filtrate prepared from a second 6 ml. aliquot of

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the plasma-substrate mixture added to 10 ml. of 0.3N trichloracetic acid, and the difference between the two values was used to indicate the quantity of chromogenic substances produced during incubation of the plasma-substrate system. Since some acid-soluble chromogenic substances appear after prolonged incubation of the hemoglobin-substrate, a "blank" solution of distilled water and hemoglobin was treated like the plasma-hemoglobin mixture and likewise incubated for twenty-four hours. Substraction of the amount of chromogenic substances produced during the incubation of hemoglobin with water from that resulting from the incubation of the plasma-substrate system represents the "tyrosine" released by the plasma. All results are reported in terms of the amount of "tyrosine" released by the proteolytic action of 1 ml. of plasma.

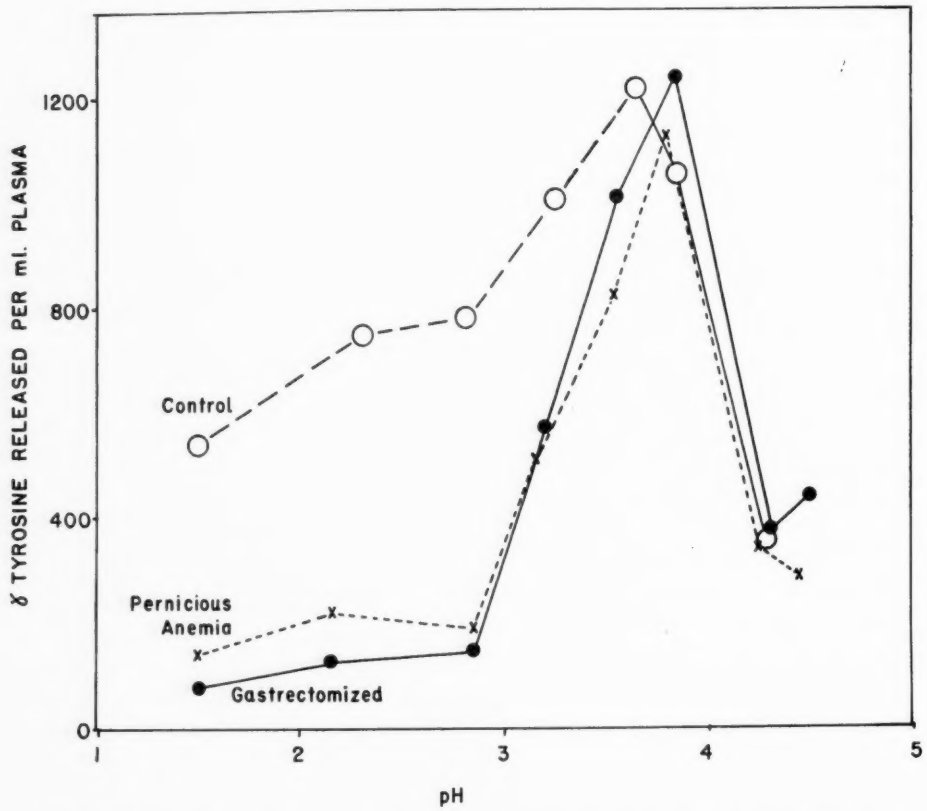

Fig. 1.—pH activity of plasma from healthy subjects, patients with pernicious anemia, and patients with total gastrectomy. The proteolytic activity is expressed as micrograms of "tyrosine" released by the action of 1 ml. plasma during standard assay.

RESULTS

In order to demonstrate the presence of a proteolytic enzyme in the blood plasma which is active at acid reactions and which is dependent upon the presence of a functioning gastric mucosa, pH activity curves were constructed with plasma of blood samples from healthy subjects, men with total gastrectomy, and patients with pernicious anemia (Fig. 1). Analysis of these curves re-

*It is essential that precautions are taken to prevent hemolysis, since erythrocytes contain proteolytic enzymes which are active at acid reactions.[15]

vealed the presence of at least three proteolytic systems which are active in the acid range in plasma of healthy men. One of the proteolytic systems is active at the optimal pH for the peptic digestion of hemoglobin (pH 1.5 to 3.0), and appears to be dependent upon the function of an intact gastric mucosa since most of the activity at this reaction is lost in the absence of the stomach or in pernicious anemia. A second proteolytic system with maximal activity between pH 3.5 to 4.0 does not require the presence of functioning gastric mucosa since its activity is affected neither by gastrectomy nor pernicious anemia, either of which would abolish that system originating from the normal intact stomach.

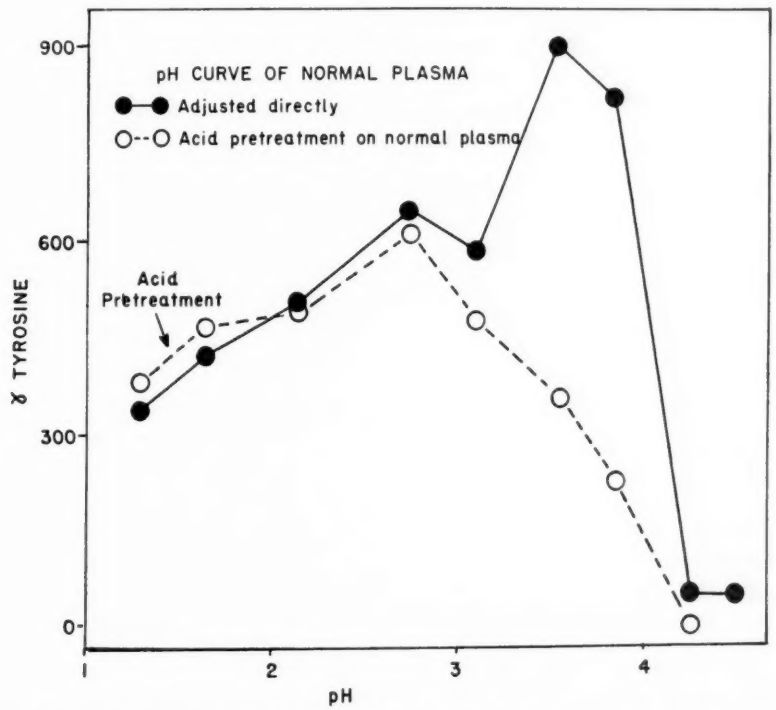

Fig. 2.—Effect of preliminary incubation at pH 1.5 on pH activity curve of plasma from healthy subject. Activity is expressed as micrograms of "tyrosine" released by the action of 1 ml. plasma during standard assay.

In addition to these systems, as will be demonstrated later, is one which is responsible for the activity at pH 1.5 to 3.0 in the plasma from the completely gastrectomized patient.

Cathepsins from animal tissues can be distinguished from pepsin by their rapid inactivation at highly acid reactions.[17] For this reason, plasma-hemoglobin mixtures were first adjusted to pH 1.5 and incubated for one-half hour at 3° to 5° C. and then readjusted to from pH 1.25 to 5 and their proteolytic activity determined in the described manner. This preliminary adjustment to pH 1.5 ("acid pretreatment") produced no significant change in the activity of the plasma at pH 1.5 to 3.0, but markedly reduced that at pH 3.5 to 4.0 (Fig. 2).

Similar results were obtained with plasma from subjects with duodenal ulcer, total gastrectomy, and pernicious anemia. This suggests that the system with maximal activity at pH 3.5 to 4 may be derived from cathepsins originating in tissues other than the stomach.

Since pepsin is inactivated rapidly at the pH of blood,[18] and since the intravenous administration of this enzyme to dogs does not result in an increase in the proteolytic activity of urine at pH 1.5,[3] it appeared probable that the

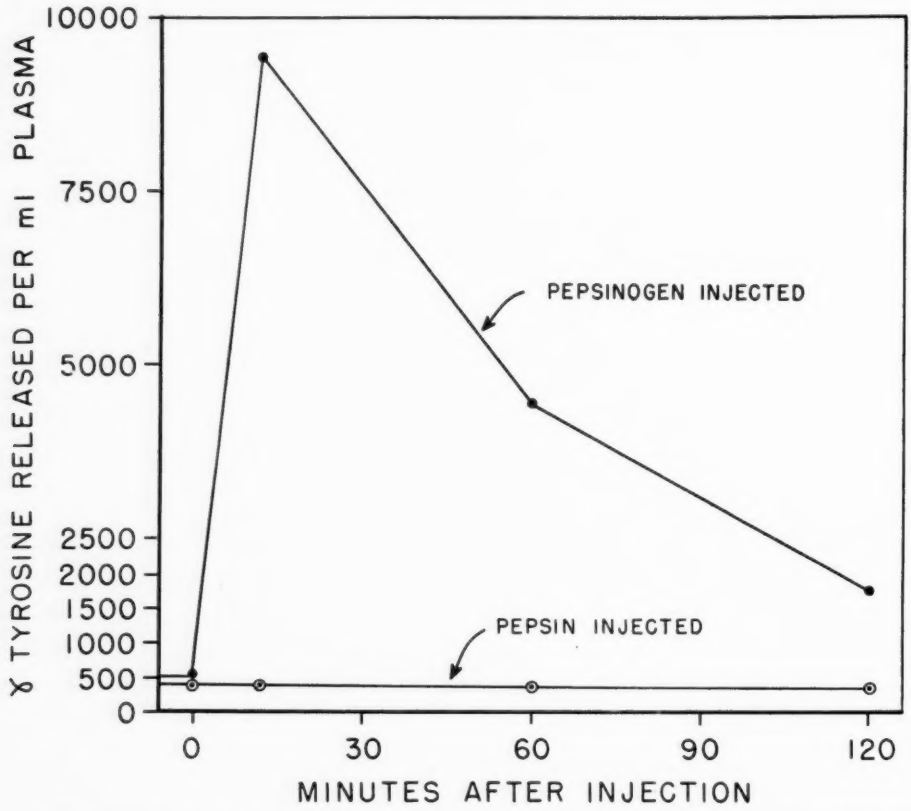

Fig. 3.—Effect of intravenous injection of crude pepsinogen and pepsin on the proteolytic activity of dog plasma at pH 1.5. Crude pepsinogen was prepared from hog stomach (18) and 1.5 ml. per kilo were administered intravenously to a dog. An equal quantity was injected into another dog after preliminary acidification and conversion of the pepsinogen to pepsin. The activity is expressed as micrograms of "tyrosine" released by the action of 1 ml. plasma during standard assay.

major portion of the proteolytic activity of the plasma at pH 1.5 to 3 is due to the autocatalytic conversion of pepsinogen to active pepsin at the low pH. In accord with this hypothesis is the observation that the intravenous administration of crude hog pepsinogen into the dog results in a marked increase in the proteolytic activity of the blood plasma at pH 1.5 (Fig. 3), as well as in that of the urine at pH 1.5.[3] The injection of an equivalent amount of pepsin, however, does not produce a significant increase either in the activity of the plasma (Fig. 3) or in the subsequent activity of the urine.[3]

In contrast to pepsinogen, approximately 80 per cent of the activity of pepsin is destroyed at pH 7.5, and almost 100 per cent is destroyed at pH 10. Consequently, if the proteolytic activity of plasma at pH 1.5 is due to the autocatalytic conversion of pepsinogen to pepsin, then plasma which has been first adjusted to pH 1.5 should react like pepsin. To test this possibility, samples of plasma from healthy subjects, patients with pernicious anemia, and patients with duodenal ulcer were added to the hemoglobin substrate, adjusted to pH 1.5, and incubated at 37° C. for one-half hour. Subsequently, one aliquot was assayed directly and another adjusted to pH 7.5 or pH 10 and then incubated for one-half hour at 37° C., after which interval they were adjusted back to pH 1.5 and comparable volumes assayed for proteolytic activity.

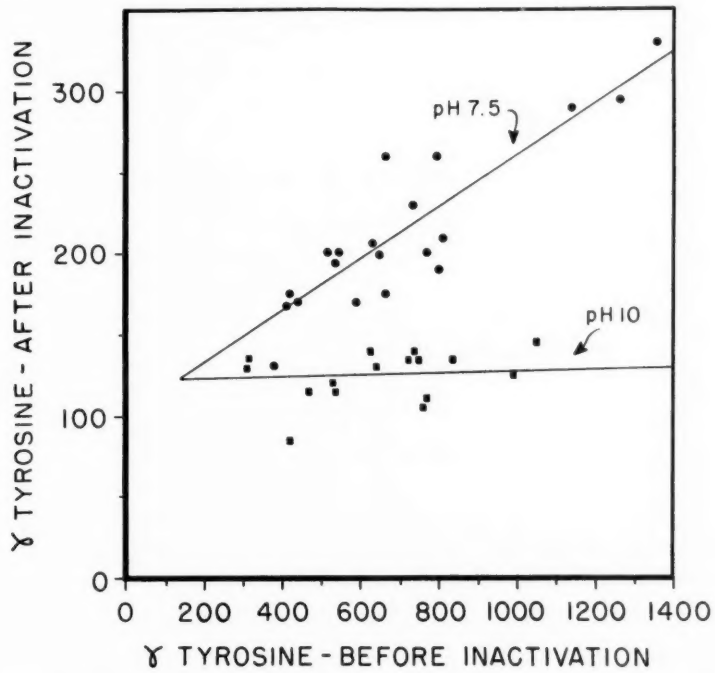

Fig. 4.—Effect of alkalinization on the proteolytic activity of acidified plasma. The activity is expressed as micrograms of "tyrosine" released by the action of 1 ml. plasma during standard assay.

The proteolytic activity of plasma at pH 1.5 before and after incubation at pH 7.5 and pH 10 is depicted in Fig. 4. It is apparent that alkalinization reduced the activity of the plasma and that the inactivation was greater at pH 10 than at pH 7.5. The regression coefficients of the activity after alkalinization on that before such treatment is 0.165 for the pH 7.5 treatment and 0.0055 for the pH 10 treatment. Accordingly, it may be stated that for every increment of 100 micrograms of "tyrosine" released by 1 ml. of plasma at pH 1.5 before alkalinization, an additional 16.5 micrograms are released after treatment at pH 7.5 (83.5 per cent inactivation) and an additional 0.6 microgram is released after treatment at pH 10 (99.4 per cent inactivation). It is apparent also that

the point at which the slopes for the pH 7.5 and pH 10.0 treated plasmas intersect represents the quantity of proteolytic activity at pH 1.5 per millimeter of plasma (i.e., 145 γ) which cannot be attributed to pepsin.

Although the major portion of the proteolytic activity of normal plasma at pH 1.5 to 3.0 can be attributed to the autocatalytic conversion of pepsinogen to pepsin, there is an appreciable activity which is independent of the presence of the stomach. In man, total gastrectomy results in a reduction of the proteolytic activity at pH 1.5, so that from 100 to 200 micrograms of "tyrosine" are released from the substrate by 1 ml. of plasma. In the dog, the activity at

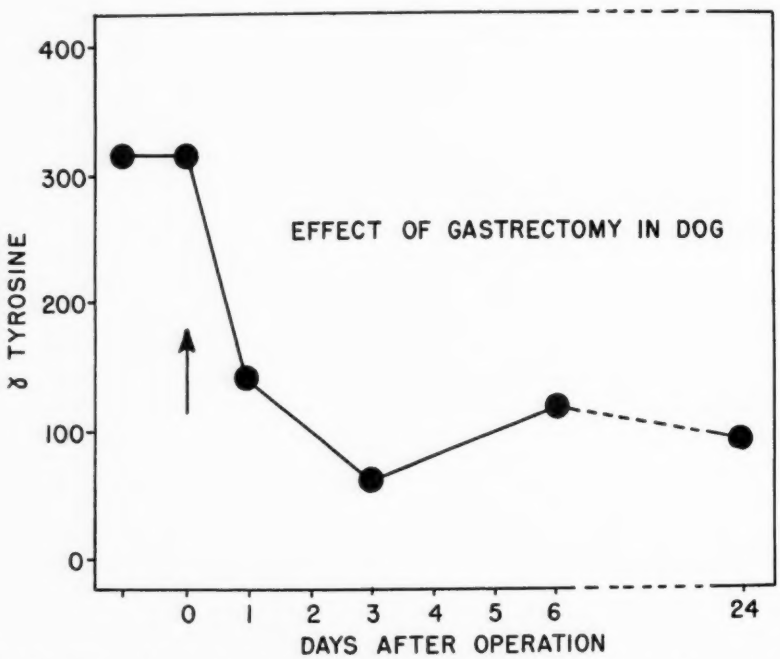

Fig. 5.—Effect of total gastrectomy on the proteolytic activity of dog plasma at pH 1.5. Expressed as micrograms of "tyrosine" released by the action of 1 ml. plasma during the standard assay.

pH 1.5 falls off rapidly after total gastrectomy and becomes stabilized at a level where 100 to 150 micrograms of "tyrosine" are released from the hemoglobin substrate by 1 ml. of plasma (Fig. 5). Likewise, the data on the effect of alkalinization of acidified plasma suggest that, of the total amount of chromogenic substances resulting from the action of 1 ml. plasma on hemoglobin at pH 1.5, approximately 150 micrograms are due to the action of an enzyme other than pepsin (Fig. 4).

The applicability of the procedure described herein for the assay of the proteolytic activity of plasma at pH 1.5 is indicated by the linear relation between the degree of proteolysis at this reaction and period of incubation at 37° C. (Fig. 6), as well as by the linear relation between the degree of proteolysis and the quantity of plasma (Fig. 7).

TABLE I. RECOVERY OF ADDED PEPSIN

PREPARATION	ACTIVITY OF PLASMA AT pH 1.5*	
Plasma	516	446
Pepsin†	654	700
Plasma + Pepsin‡	1028	1078
% recovery of activity of added pepsin	87.9	94.1

*Expressed as micrograms of "tyrosine" released by activity of 1 ml. of preparation during standard assay.

†0.1 microgram crystalline pepsin (Armour's) per milliliter water.

‡0.1 microgram pepsin per milliliter acidified plasma.

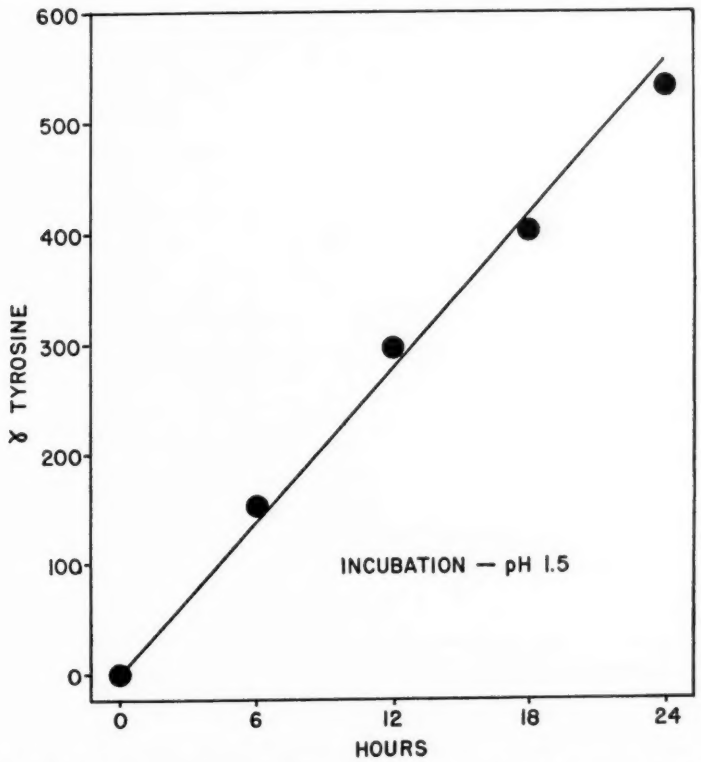

Fig. 6.—Relation between the quantity of "tyrosine" released by action of 1 ml. plasma at pH 1.5 and the length of incubation at 37° C.

In order to determine whether some inhibitor of pepsin might play a role in the activity of plasma at pH 1.5, crystalline pepsin was added to acidified plasmas of known activity (Table I). Such experiments revealed a recovery of approximately 90 per cent of the added pepsin, thereby indicating that inhibitors play an insignificant role and that the procedure assays the total pepsin content. The accuracy of the assay is evident from analyses of the differences between results obtained from duplicate samples which revealed a standard deviation of 7.4 per cent from the mean values.

Although the stability of pepsinogen in the urine has been established,[3] it was essential to determine that the activity of plasma behaved similarly and did not diminish with such periods of time as may elapse between the withdrawal

TABLE II. STABILITY OF PROTEOLYTIC ACTIVITY OF PLASMA AT PH 1.5*

CONDITION OF STORAGE	DURATION OF STORAGE		
	0 HR.	24 HR.	48 HR.
Cold room (3° to 5° C.)	1010	958	980
Room temperature	776	710	710
Room temperature	320	313	360

*Expressed as micrograms of "tyrosine" released by activity of 1 ml. plasma during standard assay.

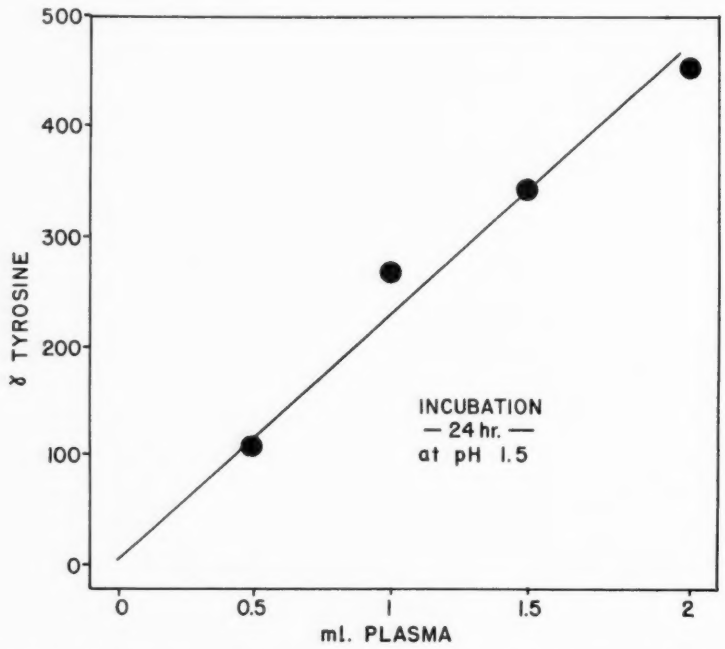

Fig. 7.—Relation between the quantity of "tyrosine" released and quantity of plasma incubated at pH 1.5 for twenty-four hours at 37° C.

of the specimen of blood and its analysis. Blood samples taken from various subjects were centrifuged and the plasma separated as rapidly as possible. One aliquot was assayed immediately, and others at intervals after the plasma was stored either at room temperature or at 3° to 5° C. in a cold room. Table II reveals that no significant change occurred during forty-eight hours' storage. Similar results were obtained with samples stored for ninety-six hours.

DISCUSSION

It is apparent from the studies reported herein that plasma from healthy subjects probably contains at least three systems with proteolytic activity at acid reactions. One system, active at pH 3 to 4, is not dependent on the presence of the stomach and is inactivated at pH 1.5. A second system, which is also independent of the stomach, is responsible for a minor fraction of the total proteolytic activity of plasma at pH 1.5 to 3 and is not affected by manipulations which inactivate pepsin. A third system, with maximal activity at pH 1.5 to 3,

is responsible for the greater part of the total activity at this reaction, is derived from the stomach, and resembles pepsinogen in its physicochemical characteristics.

Since the major portion of the activity of normal plasma at pH 1.5 to 3 is due to the system which is dependent upon the presence of a functional gastric mucosa and which is inactivated by alkalinization, and since pepsin is rapidly destroyed in the blood stream whereas pepsinogen is not, it appears highly probable that it is pepsinogen that is secreted by the peptic cells of the stomach into the circulation[3] and that with acidification "in vitro" an autocatalytic conversion to pepsin ensues. These and other considerations[7, 8] support the hypothesis that a portion of the pepsinogen manufactured by the peptic cells is excreted into the lumen of the stomach, and a portion is secreted into the circulation.

The fact that approximately 150 micrograms of the chromogenic substances released by the action of 1 ml. of plasma at pH 1.5 is due to some enzyme other than pepsin raises the question as to whether the procedure described herein can be utilized for the assay of pepsinogen in plasma. This problem can be resolved by assaying the proteolytic activity of acidified plasma before and after inactivation at pH 10, since the difference represents the activity due to pepsin. Such a procedure, however, involves the use of larger quantities of plasma and complicates the assay considerably. On the other hand, the constancy of the quantity of activity attributable to the nonpeptic enzyme (Fig. 4) suggests the possibility of either correcting each assay by subtracting 150 to 200 micrograms from the total quantity of "tyrosine" released per milliliter of plasma or disregarding it entirely.

SUMMARY

1. Plasma from healthy subjects contains at least three systems which display proteolytic activity at acid reactions. One system, active at pH 3.5 to 4, is independent of the presence of the stomach and is inactivated at pH 1.5. A second system, active at pH 1.5 to 3, is independent of the presence of the stomach and is not affected by alkalinization. A third system is dependent upon the presence of functioning gastric mucosa and is inactivated by alkalinization.

2. The major proportion of the activity of plasma at pH 1.5 is due to the system which behaves like pepsin and its presence is attributed to the autocatalytic conversion of pepsinogen.

3. A procedure for the assay of plasma pepsinogen is described.

4. The data confirm the concept that the peptic cells of the stomach have both endocrine and exocrine functions.

The technical assistance of Miss Doris Riedinger is gratefully acknowledged.

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PROBLEMS IN THE MANAGEMENT OF ERYTHROBLASTOSIS FETALIS, WITH FIVE EXAMPLES EXHIBITING UNUSUAL SEROLOGICAL FINDINGS

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DURING the past few years there have been important advances in the knowledge of the Rh-Hr types, and new sensitive and specific methods have been developed for detecting and titrating Rh and Hr antibodies. The heredity of the Rh-Hr types has been satisfactorily elucidated on the basis of a series of multiple allelic genes, and a simple terminology for designating this complicated system of blood types is available. With the newer tests it has become possible to diagnose accurately virtually all cases of erythroblastosis due to Rh or Hr sensitization. In fact, erythroblastosis has become unique in that it is probably the only disease which can be diagnosed or excluded with almost 100 per cent certainty by means of laboratory tests alone, without examining the patient. Indeed, when accurate antenatal tests are carried out, the diagnosis can usually be made before the baby is born.[1]

Correlated with the serological advances, there have been changes in the management of the disease, notably treatment by exchange transfusion. Many articles have been published containing statistical data comparing different methods of treating such patients. In previous papers[2, 3] several statistical reports have been presented, and it is planned to make additional analyses as the number of patients treated increases. Such statistical analyses are valuable and have served to demonstrate, for example, the importance of maternal Rh antibody titer for prognosis. In a recent study on more than 200 sensitized Rh-negative mothers, Wiener, Nappi, and Gordon[4] found a progressive increase in the frequency of stillbirths and in the mortality among erythroblastotic babies born alive, the higher the titer of the maternal univalent Rh antibodies antenatally. These results confirm the theory[5] that the maternal univalent antibodies are the primary cause of the disease, and are therefore the most important factors determining its severity.

While statistical studies are essential, a great deal can be learned from the thorough study of individual cases. Thus, despite the correlation between antibody titer and severity, the manifestations in different patients are so variable that it is not possible to make an absolute prediction of the outcome in any given case. In addition, many patients offer difficult diagnostic problems from the serological standpoint, namely, those in whom blood factors other than Rh_0 are involved, and especially those with multiple sensitizations. It is instructive, therefore, to study all patients thoroughly from the serological as well as the clinical standpoint. There appears to be a dearth of articles containing detailed

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case histories, illustrating the modern management of erythroblastosis. In the present report we propose to describe in detail five unusual cases which illustrate the application of modern serological tests for the diagnosis and management of erythroblastosis.

CASE REPORTS

CASE 1.—*Erythroblastosis caused by double sensitization to factors Rh_0 and rh'. Complete recovery despite high titer of Rh antibodies in baby's serum.*—Our attention was first called to this erythroblastotic infant when he was 60 hours old. This patient was the second child in the family. His mother had never received a blood transfusion. The first pregnancy, two years previously, had yielded a full-term, normal male infant who had had neither neonatal jaundice nor anemia, and was alive and well.

The patient had seemed normal at birth, but on the second day slight pallor and icterus were noted, but no enlargement of the liver or spleen was made out. The blood count showed a hemoglobin concentration of 18 Gm. per cent, with a red cell count of 4 million per cubic millimeter, and only an occasional normablast on smear. We were not notified sooner because of a mistaken impression that the mother was Rh positive. The next day the baby was noticeably paler and the splenic edge was just pa'pable. The red blood count had dropped to 3.4 million per cubic millimeter and the hemoglobin concentration to 14 Gm. The mother's blood was then tested and it was found that she was really Rh negative. At this point one of us was called into consultation and gave the baby an emergency transfusion of 70 ml. of whole blood, group O, type rh.

Grouping and Rh-Hr tests were then carried out on the family with the following results: father, O Rh_1Rh_1; mother, O rh; brother, O Rh_1rh; patient, O rh'rh. According to these results, the mother was Rh negative, the father and brother both Rh positive, but the patient himself seemed to lack the Rh_0 factor. As has been pointed out by one of us[6] previously, however, the blood of erythroblastotic babies often gives false negative reactions in tests for the Rh_0 factor by the tube agglutination method, due to "blocking" or "complete coating" of the baby's red cells by the maternal Rh_0 antibody. In fact, when our patient's red cells were suspended in an albumin-plasma mixture, spontaneous clumping (conglutination) occurred, proving that the cells were coated; the cells also exhibited strong clumping in the direct anti-globulin test (Table I).

TABLE I. DEMONSTRATION OF RH_0 AND RH' ANTIBODIES COATING BABY'S CELLS (CASE 1) BY MIXTURES WITH ENZYME-TREATED CELLS OF SELECTED RH TYPES

ONE DROP OF SUSPENSION	MIXED WITH A DROP OF ENZYME-TREATED CELLS OF TYPE				DIRECT ANTIGLOBULIN TEST
	Rh_1Rh_1	Rh_2Rh_2	rh	rh'rh	
Baby's washed red cells	++	++	–	++	+++
Rh_1Rh_1 Treated with anti-Rh_0 blocking serum and then washed three times	++	++	–	–	+++
Rh_2Rh_2 Treated with anti-Rh_0 blocking serum and then washed three times	++	++	–	–	++±
rh'rh Treated with anti-Rh_0 blocking serum and then washed three times	–	–	–	–	–
Rh_1Rh_1, washed three times	–	–	–	–	–

Tests were then carried out for Rh antibodies on the maternal serum. These showed her to be strongly sensitized not only to the Rh_0 factor, but also to the rh' factor as follows:

- *Agglutination test* (Saline medium)
  - vs. rh' and Rh_1 cells—strongly positive, 250 units
  - vs. Rh_2 cells—weak clumping (conglutination) in a first few dilutions

Blocking test

vs. Rh_2 cells—positive, 4 units

Conglutination test (albumin-plasma method)

vs. rh′ cells—positive, 160 units

vs. Rh_2 cells—positive, 120 units

Enzyme-treated cells (using the enzyme ficin)

vs. rh′ cells—positive, 250 units

vs. Rh_2 cells—positive, 400 units

Interpretation: The tests against type rh′ cells clearly demonstrate the presence of a strong rh′ antibody in the maternal serum; this antibody was largely of the bivalent or agglutinating type, as shown by the fact that the titers obtained were approximately the same in saline and in plasma media. The reactions obtained with Rh_2 cells were due to a strong Rh_0 antibody; this antibody was predominantly of the univalent or conglutinating type, as shown by the virtual absence of clumping in tests carried out in saline media.

Tests were then carried out for free Rh antibodies on the baby's serum, and the following results were obtained:

Agglutination test (in saline media)

vs. rh′ cells—negative (or less than 8 units)*

vs. Rh_2 cells—negative

Blocking test

vs. Rh_2 cells—positive, 4 units

Conglutination test (albumin-plasma method)

vs. rh′ cells—negative (or less than 8 units)*

vs. Rh_2 cells—positive, 64 units

Enzyme-treated cells

vs. rh′ cells—positive, 96 units

vs. Rh_2 cells—positive, 400 units

Interpretation: Thus, the baby's serum, like the mother's serum, contained both rh′ and Rh_0 antibodies. The rh′ antibodies in the baby's serum differed from those present in the maternal serum in that they did not react in saline media,† and the titer against enzyme-treated cells was about half as high as that of the maternal serum. On the other hand, the Rh_0 antibody in the baby's serum was of about the same titer as the Rh_0 antibody in the maternal serum. The presence of rh′ and Rh_0 antibodies in the baby's serum as well as in the maternal serum indicated that the baby's illness was due to double sensitization of the mother to the factors Rh_0 and rh′. While the maternal rh′ antibodies were demonstrable in saline media, it appeared that the mother's serum contained a mixture of bivalent and univalent rh′ antibodies, of which the former was of higher titer, so that the latter could be demonstrated only indirectly, namely, by their presence in the baby's serum.

*Because of the limited amount of serum available, this titration was started with the 1:8 dilution.

†This confirms once more the observation made by one of us[7] that univalent antibodies pass freely through the normal placenta, while bivalent antibodies do not traverse the intact placental barrier.

The question arose whether the positive reactions for "coating" of the baby's cells were due to the Rh_0 antibody, the rh′ antibody, or both. Moreover, the failure of the baby's cells to clump in saline media with bivalent anti-Rh_0 testing serum raised the question whether this was due to "blocking" or to the fact that the baby really belonged to type rh′, as was genetically possible. If this was due to blocking of the Rh_0 reaction, it was not entirely clear why the rh′ reaction was not blocked also.* We could not settle the problem whether or not the Rh_0 agglutinogen was present in the baby's red cells by using univalent Rh_0 typing serum, because the baby's cells being coated would necessarily clump in any antiglobulin or conglutination test. The fact that the Rh_0 antibody titers in the baby's and mother's sera were approximately equal added to the confusion; in Rh-negative babies the univalent Rh antibody titer always approximates that of the mother, but in Rh-positive babies, as a rule, the titer of free Rh antibodies is usually lower than the maternal Rh antibody titer, due to adsorption of the antibody on the baby's Rh-positive cells.

To settle this problem the following experiment was done. Red cells of types Rh_1Rh_1, Rh_2Rh_2, rh, and rh′rh were treated with papain in the usual way. Separate suspensions of cells Rh_1Rh_1, Rh_2Rh_2, and rh′rh were treated with an anti-Rh_0 slide test serum in order to coat the cells with Rh_0 antibody (the rh′ cells were included as a negative control), and these coated cells as well as the baby's untreated cells were washed three times with saline solution to remove the serum proteins. One drop of a 2 per cent suspension of washed "coated" cells was then mixed with one drop of a 2 per cent suspension of enzyme-treated cells in the combinations shown in Table I. As has been demonstrated previously,[8] when Rh-positive red cells coated with Rh_0 antibodies are mixed with enzyme-treated cells, clumping occurs in combinations only with enzyme-treated cells possessing the Rh_0 factor, and the same phenomenon holds for antibodies of other specificities. However, this reaction occurs, as a rule, only when the cells are strongly coated by antibody, as was the case for this erythroblastotic baby. The fact that this baby's washed cells clumped when mixed with enzyme-treated cells possessing either Rh_0 or rh′ agglutinogen indicated that the baby's cells were coated by both antibodies. In fact, when the baby became older, it was possible to show that he actually did belong to type Rh_1.

That the titers of the Rh_0 antibodies in the maternal and baby's sera were approximately equal is explained by the high concentration of the antibody in the maternal serum. When the maternal Rh antibody titer is low, the baby's Rh-positive red cells can adsorb the antibodies as quickly as they pass through the placenta, and there often is not enough time for the free antibody titer of the baby's serum to build up to the maternal level. When the maternal Rh antibody titer is very high, however, the fetal red cells soon become completely coated after adsorbing only a small fraction of the antibodies, so that the titers on both sides of the placenta soon become approximately equal. This is the probable explanation for the paradox presented by the present case.†

*A possible explanation is the lower avidity of the rh′ antibody in the baby's serum, as shown by the fact that it reacted only with enzyme-treated cells.[8]

†In addition, it should be pointed out that serological titrations detect relative differences rather than absolute differences. Thus, a difference in titers between 16 units and 1 unit is readily demonstrable, while a difference between 128 units and 256 units is demonstrable only with difficulty or not at all, because the latter is comparable to the difference between 1 and 2 units.

From the clinical standpoint the case is remarkable because the baby was never very sick, in spite of the high level of the antibody titer. As already pointed out, there is a strong correlation between antibody titer and prognosis. According to observations[4] on more than 200 sensitized Rh-negative mothers, when the antibody titer is as high as 120 units by the albumin-plasma conglutination method, the chance that the Rh-positive fetus will be stillborn is about 30 per cent, and among liveborn babies about 40 per cent die in spite of treatment. Yet this patient became only mildly jaundiced, and the jaundice cleared within a week. Anemia developed gradually, so that the first transfusion did

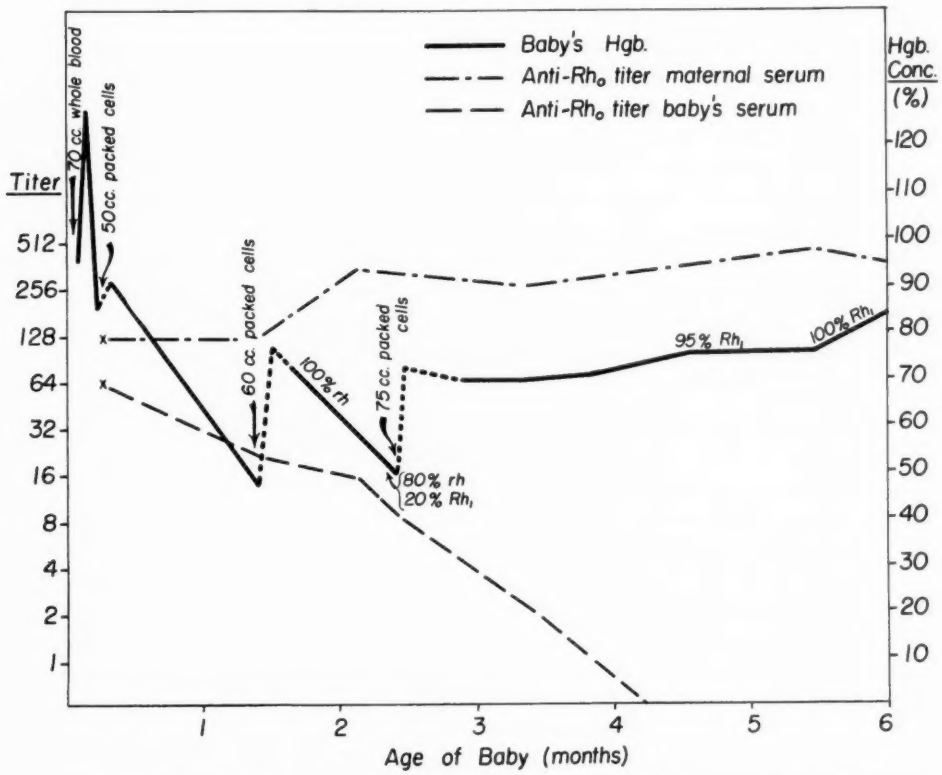

Fig. 1.—Titer of Rh$_o$ antibodies in maternal and baby's sera in relation to baby's hemoglobin concentration (100 per cent = 15 Gm.) at different ages, and differential agglutination tests on baby's cells.

not become necessary until the baby was 60 hours old. The subsequent course of the hemoglobin concentration is shown in Fig. 1. As will be seen, three supplementary transfusions of packed red cells were given, the last one when the baby was 10 weeks old.

The baby is now 13 months old, is entirely well, and has developed normally both physically and mentally. Thus, our case is similar to that of East and Mair,[9] and demonstrates that even though the Rh antibody titer is the most important consideration in prognosis, there are other factors which play a significant role in determining the course and final outcome of the disease.

Periodic titrations were performed in order to compare the changes in the titer of the antibodies in the mother's and baby's sera. In Fig. 1, the results obtained for the Rh_0 antibody titers by the albumin-plasma method are shown in parallel with the changes in the baby's hemoglobin concentration. In conformity with our past experience,[7] the mother's antibody titer exhibited no significant change during the period of observation, while the baby's titer gradually fell and became negative by the time the baby was 4 months of age. When the titrations were carried out against enzyme-treated cells, Rh_0 antibodies were demonstrated in the baby's serum for an additional two to three months. Similar results were obtained in titrations of the rh′ antibodies. In Rh-negative babies we have found that the curve of elimination of the Rh antibodies corresponds to a half-life for the antibody molecule of about 30 to 35 days.[10] The curve of elimination in this patient at first also corresponded closely to that expected under a half-life of 35 days, even though the baby was Rh positive. It is interesting to note that new Rh-positive cells appeared in the baby's circulation at a time when significant amounts of free antibodies were still present; thereafter the antibody titer fell more rapidly, probably due to adsorption by the newly formed Rh-positive cells. There seems hardly any doubt that the high titer of free Rh antibodies in the baby's serum was primarily responsible for the protracted course of the illness, so that a transfusion had to be given as late as the tenth week. Exchange transfusion therapy would probably have been more efficient, but would not necessarily have shortened the required period of observation.

Some workers have asserted that exchange transfusion shortens the course by removing large amounts of Rh antibodies. As a matter of fact, though the free antibody titer is often markedly reduced immediately after the exchange transfusion, within a day or two the original titer is restored, as a rule, by diffusion of Rh antibodies from the tissues. Apparently it is not universally appreciated that the Rh antibodies are not restricted to the blood stream but permeate the body wherever globulin-containing fluid is present. Whatever special advantages exchange transfusion may have must be attributed mainly to the removal of nondiffusible toxic materials from the blood stream, namely, the affected baby's coated cells.

CASE 2.—*Mild erythroblastosis despite high titer of Rh_0 antibodies in the maternal serum, explained by presence of an Rh_0 variant in the baby's blood.*—This patient, a male infant, was the second child in the family. During the pregnancy the parents were referred for examination because of the following history. Ten years previously the mother was treated for pneumonia, at which time she was given two transfusions of blood not tested for the Rh factor. A year previously her first baby, a girl, was delivered at term as a breech. This baby, whose blood was not available for typing, was apparently normal.

The blood of the husband reacted as group O, type M, type rh′rh. The expectant mother's blood type was A_1 MN rh′rh; she proved to be strongly sensitized to the Rh_0 factor, the titer being 1 unit by the blocking technique, 40 units by the albumin-plasma conglutination method, and 120 units for enzyme-treated cells.* Apparently, the original source of the Rh sensitization was the transfusion received ten years previously and/or the first pregnancy, but our

*The enzyme used was ficin, kindly provided by Merck and Co., Rahway, N. J.

suspicions were aroused by the high antibody titer, even though the husband seemed to be Rh_0 negative (type rh′). Therefore, additional tests were made on his blood by the antiglobulin method and after treating his cells with ficin. It was then found that he actually belonged to type $\mathfrak{R}h_1rh$, that is, his blood contained an Rh_0 variant.[11, 12] Most Rh_0 antisera failed to clump the husband's red cells even by the more sensitive techniques, but the expectant mother's serum proved to be much more active for detecting the Rh_0 variant in her husband's blood cells. It is of interest to point out that the Rh_0 variant was more readily demonstrable when the red cells were treated with ficin than by the antiglobulin method (cf. Table II). Moreover, the reactions given by the husband's cells are more reasonably explained by postulating that it contains a different *kind* of Rh_0 factor rather than a smaller *quantity* of Rh_0 factor. As shown in the table the Rh_0 factor in the husband's blood appears to react with lower avidity with Rh_0 antibodies, except when the cells are altered by treatment with proteolytic enzymes. The mother's serum proved to be a useful reagent for detecting Rh_0 variants; since she belonged to type rh′, this excluded the possibility of interference from rh′ antibodies.

TABLE II. TITRATION OF Rh_0 ANTIBODIES IN MATERNAL SERUM TO DEMONSTRATE Rh_0 VARIANT IN HUSBAND'S CELLS IN CASE 2

METHOD OF TITRATION	TEST CELLS	DILUTION OF MATERNAL SERUM								
		UNDILUTED	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Saline agglutination	Rh_2	–	–	–						
	Husband's	–	–	–						
Albumin-plasma conglutination	Rh_2	++	++	++	++	++	+±	tr.	–	–
	Husband's	±	±	±	±	±	tr.	–	–	–
Enzyme-treated cells	Rh_2	++	++	++±	++±	++	++	+±	±	–
	Husband's	++	++	++	++	++	++	+±	tr.	–
Antiglobulin method	Rh_2	++	++	++	++	++	+±	tr.	–	–
	Husband's	++	++	+±	±	–	–	–	–	–

When the baby was born, his blood reacted as A MN rh′rh. Tests for coating of the cells were negative by the conglutination method, but strongly positive by the antiglobulin method, indicating that he actually belonged to type $\mathfrak{R}h_1rh$, like his father, and was erythroblastotic. In view of the low avidity of the baby's blood cells for Rh_0 antibodies, one would expect a mild disease. In fact, the icterus index of the cord serum was only 8 units, which is within normal limits, and the blood count showed but a mild anemia, the hemoglobin concentration being 14.2 Gm. per cent, with 4.9 million red cells per cubic millimeter, and there was only one normoblast per 100 W.B.C. on the smear.

For safety the baby was given a transfusion of 60 ml. of packed type rh cells. The subsequent course was uneventful, except for the development of anemia, the hemoglobin dropping to 8.9 Gm. by the time the baby was 8 weeks old; thereafter the blood count gradually rose. The subsequent physical and mental development of the baby was normal. When the baby was 8 months old, his blood was retested and proved to belong to type $\mathfrak{R}h_1rh$, as expected.

In the baby's cord, serum free Rh antibodies were present in approximately the same titer as the mother's. Therefore, if the blood used for transfusing the baby had been Rh positive, a severe hemolytic reaction might have occurred.

CASE 3.—*Erythroblastosis resulting from double sensitization to factors rh″ and hr′.*—This patient, a male infant, was referred to us at the age of 5 days because he had been observed to become progressively more jaundiced and anemic. The patient was the third child in the family. His mother had never received a blood transfusion. The first baby had been entirely normal at birth, and was alive and well. The second baby had been jaundiced for the first month of life, but recovered without treatment and was alive and well. The patient himself seemed normal at birth, but by the time he was referred to us on the fifth day, the hemoglobin concentration had dropped to 10.5 Gm. per cent, with a red blood count of 3 million. The icterus index of his serum was 207 units, and the bilirubin concentration 20 mg. per cent.

Grouping and Rh-Hr testing of the entire family gave the results shown in Table III.

TABLE III. BLOOD GROUPS AND RH-HR TYPES OF FAMILY IN CASE 3

BLOOD OF	A-B-O GROUP	M-N TYPE	Rh-Hr TYPE	
			PHENOTYPE	GENOTYPE
Father	O	M	Rh_2Rh_2	R^2R^2 *or* R^2r''
Mother	O	MN	Rh_1Rh_1	R^1R^1 *or* R^1r'
1st child	O	M	Rh_ZRh_0	R^1R^2, R^1r'', *or* R^2r'
2nd child	O	MN	Rh_ZRh_0	R^1R^2, R^1r'', *or* R^2r'
3rd child (patient)	O	MN	Rh_ZRh_0	R^1R^2, R^1r'', *or* R^2r'

As shown in the table, all members of the family belonged to group O and were Rh positive. However, the mother belonged to type Rh_1Rh_1 and the father to type Rh_2Rh_2; the children necessarily all belonged to type Rh_ZRh_0.* Tests for abnormal isoantibodies in the maternal serum gave the results shown in Table IV. In agglutination tests in saline media, the maternal serum clumped cells of type Rh_2 (and other bloods containing the rh″ factor), indicating the presence of an agglutinin of specificity anti-rh″. By the conglutination method, on the other hand, all bloods, except those lacking the hr′ factor as well as the rh″ factor, were strongly clumped, indicating that the mother was also sensitized to the hr′ factor. Thus, the mother was doubly sensitized to factors rh″ and hr′, as in a previous case reported by us.[13] As shown in Table IV, the baby's serum on admission contained free hr′ antibodies readily demonstrable against enzyme-

TABLE IV. TITRATION OF ANTIBODIES IN MATERNAL AND BABY'S SERA IN CASE 3

SERUM OF	TIME OF TEST	METHOD OF TITRATION	TITERS FOR CELLS OF TYPES			
			Rh_1Rh	rh	Rh_2Rh_2	Rh_1Rh_1
Mother	On admission (5 days postpartum)	Saline agglutination	0	0	8	0
		Albumin-plasma conglutination	16	16	24	0
		Enzyme-treated cells	40	190	250	0
	10 days post partum	Saline agglutination	1†	0	80	0
		Enzyme-treated cells	48	200	400	0
Baby	On admission	Saline agglutination	0	0	0	0
		Albumin-plasma conglutination	0	0	0	0
		Enzyme-treated cells	2	40	48	0

†This weak reaction probably represents conglutination produced by the hr′ antibody.

*They could also belong to phenotype rh_yrh (genotype r′r″), but this alternative possibility would be very rare indeed.

treated cells. To determine whether or not rh″ antibodies were also present, either cells of type Rh_zRh_1 would have to be used or adsorption experiments carried out as in our previous case. Unfortunately, neither of these procedures could be carried out in the present case.

This baby was treated with two transfusions of packed red cells of group O Rh_1Rh_1. Recovery was uneventful, and subsequent physical and mental development has been normal.

CASE 4.—*Erythroblastosis due to rh″ sensitization in a baby of an Rh-positive mother.*—One of us was requested to see the patient, a female infant, when she was 21 hours old, because of marked jaundice and anemia. The patient was the result of her mother's third pregnancy. The mother had never received any blood or plasma transfusions and her first two babies, both girls, had been normal at birth and were alive and well. When examined, the patient exhibited considerable jaundice, edema of the eyelids, pallor, and the spleen and liver were moderately enlarged. The baby was alert and the cry was good. A blood count showed: R.B.C. 2.3 million; a hemoglobin concentration of 10.5 Gm.; W.B.C. (corrected), 38,000; polys, 68 (15 bands); lymphs, 27; monos, 4; eos, 1. Study of the blood smear showed frequent macrocytes and polychromatic red cells, reduction in the number of platelets, and 33 normoblasts per 100 W.B.C. Clinically, therefore, this was a typical case of erythroblastosis, but the mother had been reported to be Rh positive.

Grouping and Rh-Hr tests were carried out on the family with the following results: father O Rh_2rh, mother O Rh_1rh, first daughter O Rh_zRh_0 second daughter O Rh_2rh, and the patient herself O Rh_2rh. This confirmed the report that the mother was Rh positive, and suggested that she might be sensitized to the rh″ factor, which was lacking from her blood but present in the blood of the father and patient. In fact, the baby's cells were clumped by the mother's serum, and more detailed examination revealed that the latter contained anti-rh″ agglutinins with a titer of 32 units. Accordingly, the baby was given a transfusion of 80 ml. of O Rh_1Rh_1 blood.

The following day the baby appeared more jaundiced, but the cry was good, and the baby appeared alert and well fed. The hemoglobin concentration had risen to 15 Gm., R.B.C. to 4.6 million, and there were now only fifteen nucleated red cells per 100 W.B.C. The next day the jaundice increased and the baby seemed somewhat lethargic, but there were no significant changes in the hematological findings. Thereafter the baby exhibited clinical improvement, but the deep jaundice persisted for four to five days, after which it gradually subsided. At the same time the red blood count and hemoglobin concentration gradually fell, and on the sixth day of life another transfusion of 90 ml. of O Rh_1Rh_1 blood was given from the same blood donor. Again the blood count rose this time from 12 Gm. to 14.5 Gm., with a 4.7 million red cell count. Thereafter the blood count gradually fell, and when the baby was seen at the age of one month she exhibited a muddy pallor, although she appeared and behaved well otherwise. A third transfusion was therefore given, this time 80 ml. of packed red cells from an O rh donor. The blood count rose to 15 Gm. with 4.7 million red cells, and thereafter the blood count remained within normal limits, so that no further transfusions were required. At the present time this child is 22 months old and her physical and mental development has been normal and is comparable to that of her two older sisters.

CASE 5.—*Fatal erythroblastosis in the baby of an Rh-positive woman of type Rh_zRh_1, with hr′ antibodies in her serum.*—This patient, a male infant, was the result of his mother's fourth pregnancy, and was observed to be deeply jaundiced and anemic shortly after birth. The mother had received one transfusion six years previously, which she believed was plasma. Her first two babies, both boys, had exhibited no abnormalities at birth, and were alive and well, 6 and 5 years old, respectively. The third baby, a female, was delivered at term as a breech and died after 6 hours, cause unknown. When the patient was first seen by us, he was one day old, deeply jaundiced, and apparently moribund. No information was available concerning the Rh type of the mother, and a small sample of the baby's blood proved to be chocolate color and hemolyzed, and could not be typed. The baby died before any treatment could be instituted, and a blood count taken shortly before death showed: hemoglobin concentration, 7.3 Gm.; R.B.C., 2.3 million; W.B.C. (corrected), 35,000; polys, 68 (30 bands); lymphs, 22; monos, 8; eos, 2; and 150 early and 360 late erythroblasts per 100 W.B.C. were seen on the smear.

TABLE V. GROUPING AND RH-HR TYPES OF FAMILY 5

BLOOD OF	A-B-O GROUP	M-N TYPE	RH-HR TYPE	
			PHENOTYPE	GENOTYPE
Father	O	MN	Rh_2Rh_2	R^2R^2 or R^2r''
Mother	B	MN	Rh_zRh_1	R^zR^1, R^zr', or R^1r^y
1st child	O	M	Rh_zRh_2	R^zR^2, R^zr'', or R^2r^y
2nd child	B	M	Rh_zRh_2	R^zR^2, R^zr'', or R^2r^y

Grouping and Rh-Hr tests in this family gave the results shown in Table V. It will be seen that the entire family is Rh positive, but the family is unique, because this particular combination of rare Rh types has not been encountered previously. The type of the husband, Rh_2Rh_2, has an incidence of only 2 to 3 per cent among Caucasoids, while the mother's type Rh_zRh_1 is even rarer, namely, once among about 500 individuals. Thus, this particular mating would be encountered only once among about 25,000 families. The types of the two sons, Rh_zRh_2, are even rarer than that of the mother.

The fact that the mother was hr′ negative, as indicated by her type Rh_zRh_1, suggested that she might be sensitized to the hr′ factor. Indeed, her serum was found to contain univalent hr′ antibodies with a titer of 8 units by the albumin-plasma conglutination method. Recently, we retested this serum against enzyme-treated cells, by which method we obtained a titer of 50 units.

COMMENT

Cases 3, 4, and 5 constitute a serological trilogy. In one case the mother belonged to type Rh_1Rh_1, so that she lacked two factors, rh″ and hr′, and became sensitized to both; in the second case, the mother belonged to type Rh_1rh, so that she lacked the rh″ factor but not the hr′ factor, and she became sensitized only to the former; in the third case the mother belonged to type Rh_zRh_1, so that she possessed the rh″ factor but not the hr′ factor, and she became sensitized only to the latter. The counterpart of this trilogy involving factors rh′ and hr″ no doubt exists, but must be considerably rarer. In the second trilogy the types of the mothers would be Rh_2Rh_2, Rh_2rh, and Rh_zRh_2, respectively. From the clinician's point of view, such unusual cases serve to re-emphasize the fact that typical erythroblastosis can occur in a baby, even though the mother is Rh positive, based on sensitization to Rh-Hr antigens other than Rh_0.

Cases 1 and 2 are of interest because they illustrate that the Rh_0 antibodies passively acquired by placental transfer from the mother may persist in significant amounts in the baby's circulation for many months when the maternal antibody titer during pregnancy has been high. Case 1 was unusual because the mother was doubly sensitized to factors Rh_0 and rh′, and both these antibodies were demonstrated coating the baby's cells and free in his circulation. Case 2 is a rare one because the Rh_0 factor in the cells of the father and baby was a variant that could be demonstrated only with special anti-Rh_0 sera, including that of the mother, and then only when the cells were enzyme-treated or by the antiglobulin technique. The mildness of the symptoms despite the high titer of the Rh_0 antibodies in the mother's serum was not unexpected in view of the low avidity of the baby's cells for Rh antibodies. There is evidence that the counterpart of this situation also exists, namely, erythroblastotic babies with Rh-positive cells of average avidity, whose sensitized mothers have Rh_0 antibodies of low avidity. By taking the question of avidity into account, it will no doubt be possible to explain some of the exceptional instances, where a baby is severely affected despite a low titer of Rh_0 antibodies in the maternal serum, as well as the opposite, where the baby is mildly affected despite a high titer in the mother. Low avidity is no doubt also the explanation of the mildness of the symptoms in the babies of mothers sensitized to the A and B agglutinogen. The low avidity here is due in part to the heterogenetic origin of the natural alpha and beta antibodies,[14] and in part to the incomplete state of development of the A-B-O agglutinogens in the fetus and newborn.[15]

SUMMARY

Five clinically typical cases of erythroblastosis fetalis with unusual serologic findings are presented. The first case involves a type rh woman strongly sensitized to the Rh_0 and rh′ factors, in which both these antibodies were demonstrable coating the affected baby's cells and free in his serum. In the second case the baby was mildly affected despite a high titer in the maternal serum; this was explained by the demonstration that the baby's Rh_0 factor was a variant of low avidity. The last three cases form a trilogy; one mother belonged to type Rh_1Rh_1 and was doubly sensitized to factors rh″ and hr′; the second mother belonged to type Rh_1rh and was sensitized to factor rh″; while the third mother belonged to type Rh_2Rh_1 and was sensitized to factor hr′.

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THE UTILIZATION OF FRUCTOSE* BY HUMAN SUBJECTS AND ANIMALS

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IT HAS been reported that invert sugar is retained more readily and in greater amounts by the body than glucose at either slow or rapid rates of intravenous infusion.[1-4] Determinations of the total reducing substances and the fructose in the blood and urine of individuals receiving intravenous invert sugar (a 1 to 1 mixture of glucose and fructose) showed: (a) Fructose was removed more rapidly from the blood stream than glucose. (b) A more rapid removal of the glucose than when similar amounts of glucose were given alone at the same rate. (c) A bilaterally enhanced utilization of glucose and fructose when they were administered as invert sugar.

To elucidate our previous observations that fructose is removed more rapidly from the blood than glucose when given intravenously as an invert sugar preparation,[5] we have studied the effect of intravenous infusions of fructose in human subjects. We also made a quantitative study of the uptake of fructose by the liver in rabbits and dogs, and by muscle in dogs. The latter experiments help to explain some of the clinical findings to be elucidated and previously reported by others.

MATERIALS AND METHODS

Human Experiments.—Nonoperative patients on the surgical wards or convalescing operative patients were used for the tolerance tests. Each patient was given 47 or 50 Gm. of fructose dissolved in 1,000 ml. of water as a single controlled-rate intravenous infusion. The test was conducted in the following manner: (a) No food was permitted after the evening meal of the day before the experiment, and oral water was restricted after midnight of the day of the experiment. Hence, each patient was in a postabsorptive state. (b) The individuals were weighed prior to the infusion, and the rate of infusion for each 1,000 ml. of fructose solution was calculated. A rate of 0.75 Gm. of fructose per kilogram of body weight was selected. The infusions were given intravenously by the drip method; the exact time required for the injection was recorded by the technician, who remained with the patient throughout the entire period of infusion. From the exact time required for the intravenous injection and the weight of the patient, the true rate of administration was calculated for each experiment. (c) Urine was collected by voluntary voiding before the test, after the infusion of 500 ml. of solution, after 1,000 ml. of solution, and one, two, and three hours after the end of the infusion. If the three-hour urine sample, after the end of the infusion, gave a positive Benedict's test, a four-hour urine specimen was obtained and included in the study. In most instances, the four-hour sample was not necessary. No fluids or food were permitted, and the patients were kept in bed throughout the entire test period. The urine specimens were tested for specific gravity, grams of total reducing substance by Somogyi's method,[6] and grams of fructose by Roe's method.[7] (d) Blood samples were drawn from

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the arm not used for the infusion at the same time intervals as described above for the collection of the urine specimens. Blood samples were analyzed for total sugar by the Somogyi procedures and for fructose by Roe's[7] method. The hematocrit value was determined with Wintrobe tubes. (e) Oral temperatures and blood pressure readings were noted before and at the end of the test period. Ages were also recorded.

Animal Experiments: Fructose Uptake By The Liver.—Experiments were carried out in which the concentration of fructose in the blood of the portal and hepatic veins was determined after the administration of 5 Gm. of fructose per kilogram of body weight in four rabbits and one dog. Light anesthesia was produced by injecting intraperitoneally 30 mg. of Nembutal per kilogram of body weight, and surgical anesthesia was established by the use of ether when operative procedures were carried out. A laparotomy was performed on the animal, and the fructose solution was injected directly into the duodenum. The abdomen was closed with clamps, and the animal was kept warm. At intervals after the injection of the fructose, blood samples were collected simultaneously from the portal and hepatic veins. The fructose content of the blood samples was determined.

Animal Experiments: Fructose Uptake By Muscle.—Dogs were anesthetized by injecting intraperitoneally 30 mg. of Nembutal per kilogram of body weight. Additional small amounts of Nembutal were injected as needed through the experiment. A solution containing 3 Gm. of fructose per kilogram of body weight was injected intraperitoneally and subcutaneously. Blood samples were collected simultaneously from the femoral artery and the femoral vein at approximately thirty-minute intervals after injection of the fructose solution and were analyzed for fructose.

RESULTS

Human Experiments.—Twenty patients, ranging in weight from 48 to 92 kilograms, and in age from 17 to 57 years, were given 47 or 50 Gm. of fructose in 1,000 ml. of distilled water at an average rate of 0.76 Gm. per kilogram per hour. Rates of infusion were rather constant, and varied between 0.65 and 0.85 Gm. per kilogram per hour, with an average rate of 0.76 Gm. per kilogram per hour.

The effect upon the blood sugar is shown in Table I. The total reducing substances in the blood after the infusion of 25 Gm. of fructose ranged between 115 and 174 mg. per cent, and averaged 144.3 mg. per cent. At the end of the infusion, the total reducing substances of the blood averaged 143.9 mg. per cent. The latter value was thus no higher than that found after the infusion of 25 Gm. This finding shows that the rate of removal of sugar from the blood for the second 25 Gm. of fructose infused was practically equal to the rate of infusion, and is more rapid than was noted for the first 25 Gm. given. In eleven instances the estimations of total reducing substances one hour after the end of the infusion averaged 85.6 mg. per cent, or values below that noted before the infusion. Three hours after completing the infusion, the total blood sugar values were at preinfusion levels. The maximal increase in total reducing substances at the end of the infusion ranged between 8 and 95 mg. per cent, and averaged 50.2 mg. per cent.

Before the infusions there was no fructose in the blood, as would be expected in patients who have not had any food for twelve hours or more. The average blood fructose concentration was 29 mg. per cent after the infusion of 25 Gm. of fructose and 26.2 mg. per cent at the end of the infusion of 50 Gm. One hour after completing the infusion, no fructose was detectable in the blood. These data, in agreement with the total sugar findings cited above, show that

with infusions of fructose at an average rate of 0.76 Gm. per kilogram per hour, the removal of fructose from the blood is very rapid, and is faster for the second 25 Gm. infused than for the first.

The data in the last column of Table I were obtained by subtracting the fructose value at the end of the infusion period from the increase in total sugar at the same time. This yields a quantity of copper-reducing substance which apparently is glucose, formed from the conversion of fructose. (One may make this calculation if the minor differences in the copper-reducing capacities of fructose and glucose are neglected.) The average amount of apparent glucose resulting from the conversion of fructose was 24 mg. per cent at the end of the infusion. Therefore, assuming that fructose is converted into glucose, about 47 per cent of the maximal rise in blood sugar at this point is accounted for by the glucose formed from fructose by metabolic conversion.

The presence in the tissues of enzyme systems capable of promoting each step in the conversion of fructose to glucose suggests that this conversion may take place. Slein, Cori, and Cori[13] have shown that liver contains an enzyme, fructokinase, that catalyzes the formation of fructose-1-phosphate from fructose and adenosine triphosphate under proper conditions. These authors also demonstrated the presence in liver of an enzyme, phosphofructomutase, which forms fructose-6-phosphate from fructose-1-phosphate. In 1933, Lohman[9] showed that fructose-PO_4 is converted into glucose-6-PO_4 by the enzyme, phosphohexoisomerase, found in many tissues. Recently, Swanson[10] found that glucose-6-PO_4 is converted into glucose and free phosphate by liver phosphatase. Thus, by in vitro studies, good evidence has been obtained for the conversion of fructose into glucose through several intermediary steps, not necessarily including the formation of glycogen. The data in the last column of Table I are, therefore, assumed to represent the amounts of glucose formed from fructose.

In these experiments in human beings, it was shown that injected fructose is removed rapidly from the blood stream, and that the total circulating glucose is below the renal threshold level. As would be expected, very little glucose was excreted in the urine. However, we did find fructose in the urine, even though the blood levels of fructose were low, because in human beings there is a lower renal threshold for fructose than for glucose.[5]

Total reducing substances in the urine of the twenty subjects during the four-hour period averaged 2.3 Gm., with values ranging between 0.96 and 7.4 Gm. The urinary fructose accounted for approximately 77 per cent of the total reducing substances. The remaining 23 per cent of copper-reducing substances is accounted for by the nonsugar reducing substances of the urine and small amounts of glucose. In view of the fructosuria observed, the tolerance rate (rate of infusion without mellituria) for fructose is below 0.76 Gm. per kilogram per hour; however, this conclusion is based upon a qualitative characterization of sugar excretion. Quantitatively, the sugar lost in the urine after the intravenous administration of fructose is less than the losses we observed following the intravenous administration of similar amounts of glucose at similar rates.[1] These quantitative findings are due to the fact that fructose is removed

TABLE I. EFFECTS OF INTRAVENOUS INFUSION OF 50 GM. OF FRUCTOSE IN 1000 C.C. OF WATER AT 0.76 GM. PER KILOGRAM PER HOUR IN HUMAN BEINGS ON THE WHOLE BLOOD SUGAR AND WHOLE BLOOD FRUCTOSE AND MILLIGRAMS OF FRUCTOSE CONVERTED TO GLUCOSE

					WHOLE BLOOD GLUCOSE (MG. PER CENT)						WHOLE BLOOD FRUCTOSE (MG. PER CENT)				
CASE NO.	AGE (YEARS)	WEIGHT (KG.)	TIME (MINUTES)	RATE (GM./KG./HR.)	BEFORE INFUSION	AFTER 25 GM.	AFTER 50 GM.	1 HOUR AFTER INFUSION	3 HOURS AFTER INFUSION	INCREASE AT END OF INFUSION	BEFORE INFUSION	AFTER 25 GM.	AFTER 50 GM.	1 HOUR AFTER INFUSION	MG. PER CENT GLUCOSE FROM CONVERTED FRUCTOSE AT END OF INFUSION
1	53	60	65	.77	111	166	195		103	+84	0	42	25		+59
2	29	70	55	.78	90		164		94	+74	0		37		+37
3	46	73	55	.75	106	142	175		88	+69	0	12	27		+42
4	20	60	60	.83	74	115	136		80	+62	0	20	27		+35
5*	45	73	55	.70	83	127	142		103	+59	0	17	25		+34
6	37	60	65	.77	80	133	143		95	+63	0	12	35		+28
7	60	53	75	.75	88	171	183		69	+95	0	51	53		+42
8	17	66	70	.65	81	117	142		74	+61	0	20	22		+39
9*	18	60	55	.85	91	133	125	80	95	+34	0	27	22	0	+12
10	39	84	50	.71	97	160	148	66	79	+51	0	22	24	0	+27
11	45	92	45	.72	83	174	160	77	87	+77	0	28	26	0	+51
12*	37	68	55	.75	89	125	111	87	91	+22	0	20	12	0	+10
13	57	57	65	.81	103	129	111	87	93	+ 8	0	24	7	0	+ 1
14	13	48	80	.78	100	158	150	91	101	+50	0	41	31	0	+19
15	57	60	60	.83	117	155	143	100	100	+26	0	35	32	0	- 6
16*	60	60	55	.85	88	143	133		100	+45	0	35	32	0	+13
17*	33	60	60	.78	85	143	125	89	100	+40	0	45	23	0	+17
18*	17	60	65	.72	106	166	125	100	101	+19	0	41	19	0	0
19*	70	50	85	.66	111	153	133	85	113	+22	0	27	25	0	- 3
20*	39	75	50	.75	90	143	134	80	91	+44	0	33	20	0	+24
Average Values	39.6	64.4	61.2	.76	93.6	144.3	143.9	85.6	92.8	+50.2	0	29	26.2	0	+24

*Received 47 Gm. of fructose.

from the blood more rapidly than glucose and, therefore, the elevation of the blood sugar and the duration of the hyperglycemia are less after fructose infusion.

With the administration of 1,000 ml. of a 5 per cent solution of fructose in distilled water, the urinary excretion during the four-hour study period showed a loss of all of the water infused in most instances, and in eleven cases the urinary volumes were more than the 1,000 ml. infused. The average volume for total urine collected during the four-hour period was 1,165 ml. The lowest excretion was 650 ml., the highest 2,175 ml.

Further study of the relationship of urinary excretion of water of the intravenous infusions of fructose in distilled water is desirable.

Animal Experiments: Fructose Uptake by the Liver.—The results of the experiments with a fructose solution injected directly into the duodenum of four rabbits and one dog are shown in Table II. The fructose content of the blood from the hepatic vein was less than that from the portal vein in all cases. The decreases ranged from 7 to 75 mg. per cent in the rabbits, and were 48 and 61 mg. per cent in the dog. Thus, there occurred a marked withdrawal of fructose from the blood which passed through the liver of animals receiving fructose intraduodenally.

Animal Experiment: Fructose Uptake by Muscle.—The results obtained with the injection of a solution containing 3 Gm. of fructose per kilogram of body weight intraperitoneally and subcutaneously in four dogs are shown in Table III. The fructose content of the blood collected from the femoral vein was less than that from the femoral artery in all cases. The differences in the paired samples in each experiment are statistically significant ($P < 0.01$ for dogs 1 and 3; $P < 0.05$ for dog 4; and $P = 0.06$ for dog 2). These data show that fructose is utilized directly by muscle.

DISCUSSION

Our results with the infusion of 5 per cent fructose in human subjects are in agreement with our previous studies with invert sugar in which we found that fructose is removed more rapidly from the blood than glucose when

TABLE II. CONCENTRATION OF FRUCTOSE IN BLOOD SAMPLES COLLECTED SIMULTANEOUSLY FROM THE PORTAL VEIN AND A HEPATIC VEIN AFTER FRUCTOSE ADMINISTRATION

ANIMAL	MINUTES AFTER ADMINISTRATION	MG. OF FRUCTOSE PER 100 C.C. OF BLOOD		DECREASE IN HEPATIC VEIN	
		PORTAL VEIN	HEPATIC VEIN	MG.	%
Rabbit 1	30	117	59	58	49
	60	218	122	96	44
	90	187	127	60	32
Rabbit 2	30	15	5	10	66
	60	12	8	4	33
	90	37	14	23	62
Rabbit 3	30	81	75	6	7
	60	45	28	17	38
Rabbit 4	30	392	148	294	75
Dog 1	150	62	32	30	48
	180	95	37	58	61

The rabbits received 5 Gm. of fructose per kilogram of body weight by intraduodenal injection. The dog received 2 Gm. per kilogram intraduodenally two hours after a previous subcutaneous injection of 3 Gm. per kilogram.

TABLE III. CONCENTRATION OF FRUCTOSE IN BLOOD SAMPLES COLLECTED SIMULTANEOUSLY FROM THE FEMORAL ARTERY AND VEIN AFTER INTRAPERITONEAL AND SUBCUTANEOUS ADMINISTRATION OF FRUCTOSE

DOG NO.	SAMPLE NO.	MG. OF FRUCTOSE PER 100 C.C. OF BLOOD		
		FEMORAL ARTERY	FEMORAL VEIN	DIFFERENCE
1	1	86	69	17
	2	72	55	17
	3	65	54	11
	4	60	48	12
2	1	40	36	4
	2	47	43	4
	3	43	40	3
	4	43	39	4
3	1	41	31	10
	2	44	32	12
	3	29	25	4
	4	25	23	2
4	1	87	59	28
	2	65	59	16
	3*	113	72	41
	4	104	76	28

Dose, 3 Gm. per kilogram of body weight.
*Injected second dose. Total injection, 6 Gm. per kilogram.

injected intravenously as an invert sugar preparation. This paper also reveals that the renal threshold for fructose in human beings is lower than that for glucose, that a portion of the infused fructose is converted to glucose, and that fructose is rapidly utilized by human beings.

The rapidity with which the body can utilize fructose is well shown by our observation that the circulating fructose at the end of the 50 Gm. infusion (26.2 mg. per cent) is no greater than that noted after the infusion of 25 Gm. (29 mg. per cent). In the thirty-minute period required for the infusion of the second 25 Gm. of fructose, the rate of removal from the blood is approximately equal to the rate of infusion (0.76 Gm. per kilogram per hour), and the second 25 Gm. infused is utilized more rapidly than the first. Further evidence for the rapid utilization of fructose is revealed by the absence of fructose from the circulating blood one hour after the end of the infusion.

Convincing evidence for the assumption that fructose is utilized more rapidly than glucose is found in the work of Deuel.[12] With a human subject receiving orally 75 Gm. of fructose, Deuel observed a distinct rise in the respiratory quotient in the first ten minutes after ingestion, and during the second ten-minute period the respiratory quotient usually reached unity. Following the administration of the same amount of glucose, only a slight rise in the respiratory quotient occurred during the first forty-five minutes, and maximal value, which never reached unity, was not observed until the second hour after administration. These results show that, following oral administration, fructose is oxidized more rapidly than glucose in the human subject.

Further evidence to explain the rapid utilization of fructose is offered by our animal work, which shows that both liver and muscle are equipped to remove fructose from the blood stream rapidly. These experiments confirm the findings of other laboratories in which in vitro techniques were used. Slein, Cori, and Cori[13] obtained enzyme preparations from rat liver which catalyzed

the phosphorylation of fructose. The primary reaction product was fructose-1-phosphate. These authors also found that liver contains a mutase system which converts fructose-1-phosphate to fructose-6-phosphate. Vestling and associates[14] showed that in rat liver slices and homogenates, fructose phosphorylation occurred ten times more readily than glucose phosphorylation. Cori[15] found that in the rat, orally administered glucose and fructose formed liver glycogen at approximately the same rate; however, since glucose is absorbed faster than fructose, the percentage of the sugar absorbed that was deposited as liver glycogen was 2.3 to 4.8 times greater with fructose than with glucose. Thus, it has been shown that liver contains enzyme systems that transfer fructose directly into the glycolytic cycle, and that the liver forms glycogen more rapidly from fructose than from glucose.

Slein, Cori, and Cori[13] made an enzyme preparation from rabbit muscle that catalyzed the phosphorylation of fructose. These authors could not establish whether the phosphate ester was fructose-1-phosphate or fructose-6-phosphate. However, Cori and Slein[16] obtained a protein fraction from rabbit muscle which catalyzed the conversion of either fructose-1-phosphate or fructose-6-phosphate into fructose-1,6-diphosphate. Thus, it has been found that muscle also contains enzyme systems that transfer fructose directly into the glycolytic cycle. Our studies, showing that fructose is taken up directly by muscle, offer important confirmation of these in vitro findings upon fructose metabolism in muscle.

It is of considerable interest that Slein, Cori, and Cori[13] observed a more rapid utilization of glucose than fructose by muscle extract (F:G ratio about 0.1), a situation opposite to that in the liver. Thus, a comparison of rates of utilization of fructose and glucose, when made by in vitro studies, varies according to the tissue involved, and an answer to this question is obtainable only by tolerance tests upon the whole animal. The clinical importance of careful studies upon human subjects is, therefore, obvious.

The fact that we found that a portion of the fructose was converted to glucose helps in part to explain the rapid removal of fructose from the blood, and also shows the fallacy of recording only blood fructose values when determining the effect of fructose infusions on the circulating blood sugar level. The total reducing substance must be recorded simultaneously with the determinations of circulating fructose when studies with fructose are conducted.

Fletcher and Waters[17] reported no depressant effect of fructose upon the blood glucose of the fasted normal or depancreatized dog when the blood glucose concentration is low. Our data upon postabsorptive human subjects receiving intravenous fructose infusions confirm the above finding; in fact, our results show a rise in blood glucose after fructose administration.

In view of previous reports and of our data in human beings and animals, one can say that fructose is rapidly utilized, and because of its rapid oxidation is a good protein sparer and has a high antiketogenic value. Fructose also produces less hyperglycemia, and less glycosuria than equal quantities of glucose at similar rates of infusion.

These qualities of fructose have important clinical applications in both medical and surgical conditions, viz., in liver disease, in diabetes, and in post-operative ketosis. However, there is one drawback to intravenous fructose; this is its low renal threshold, and the subsequent mellituria which occurs at low blood fructose levels.

SUMMARY

Tolerance studies in human subjects receiving 47 or 50 Gm. of fructose in 1,000 ml. of distilled water at an average rate of 0.76 Gm. per kilogram per hour showed:

1. Fructose is rapidly removed from the blood. With a continuous intravenous infusion of fructose, the second 25 or 23.5 Gm. are removed from the blood at practically the rate of infusion.

2. Low levels of hyperglycemia and a rapid return to normal blood sugar levels were seen. After the infusion of the first 25 Gm. of fructose, the average value for reducing substances in the blood was 144.5 mg. per cent, and at the end of the 50 Gm. infusion, the average value was 143.9 mg. per cent. One hour later the circulating blood sugar levels were similar to the preinfusion values.

3. A portion of the injected fructose appears to be converted to glucose, and the latter is detectable in the blood during the period of infusion. Forty-seven per cent of the maximal rise in the total blood sugar at the end of the infusion apparently is due to the glucose resulting from the rapid metabolic conversion of fructose to glucose.

4. Fructosuria occurred in all cases studied. The average amount of reducing substances recovered was 2.3 Gm., or the equivalent of a loss of 4.6 per cent of the fructose infused. The range of urinary mellituria was between 0.96 and 7.4 Gm. Therefore, the tolerance rate (rate of infusion of a sugar without mellituria) for fructose is below 0.76 Gm. per kilogram per hour.

5. The observation of fructosuria associated with low levels of fructosemia indicated a low renal threshold for fructose in human subjects.

In rabbits and dogs, after the injection of fructose intraduodenally, there was a marked withdrawal of fructose from the blood passing through the liver. Estimations of the fructose in samples of blood collected simultaneously from the femoral artery and vein after the injection of 3 Gm. of fructose per kilogram of body weight in dogs showed a direct utilization of fructose by the muscle of the extremity. These data offer important confirmation of in vitro studies reported by others.

In view of the rapid utilization of fructose by human beings, its use in medical and surgical conditions which require rapid glycogen formation, low levels of hyperglycemia, active protein sparing, and antiketogenic activity, should be entertained. Fructose may prove to be a valuable carbohydrate in the management of the diabetic patient, and in the treatment of the patient with low glycogen reserves.

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CHANGES IN BACTERIAL SENSITIVITY TO AUREOMYCIN AND CHLORAMPHENICOL IN THE COURSE OF THE PAST THREE YEARS

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A PRIME problem following the introduction of new chemotherapeutic agents has been the appearance of resistant strains from originally sensitive bacteria after a varying period of enthusiastic use. This complication has developed with such rapidity and to so marked a degree with the sulfonamides and with streptomycin that their clinical value is now extremely limited. So far this has not been true with penicillin, with one exception. Numerous recent reports in the literature[1-5] attest to a striking progressive increase in the percentage of penicillin-resistant staphylococci isolated as compared to the initial period following the discovery of penicillin when almost all strains had been sensitive. Potentially this might have produced a serious therapeutic problem, but, fortunately, other effective antibiotics or combinations of drugs are still available to combat successfully these infections.

The so-called "broad spectrum" antibiotics have now been employed on a wide scale for approximately three years. In the light of the above experiences, it became of timely interest to determine whether similar changes in bacterial sensitivity have developed since the introduction of these newer antimicrobial agents. Microorganisms isolated from a variety of pathological sources, e.g., blood, urine, spinal fluid, stool, sputum, abscesses, and purulent exudates, are promptly subjected to sensitivity tests to a number of different antibiotics in this laboratory. This afforded an excellent opportunity to compare the relative susceptibility of different bacteria to the newer drugs in the course of the past three years. Only those organisms obtained on primary isolation immediately following admission to the hospital are included in this survey. Although earlier treatment with one of these antibiotics prior to hospital admission might conceivably have altered the bacterial sensitivity of some of these organisms, information regarding this factor has been unobtainable in the great majority of cases, and has been omitted of necessity. It was decided to limit this survey to aureomycin and chloramphenicol, since terramycin has been in general use for only a year and a half; and it has been shown[6-10] that with rare exceptions natural and acquired resistance of most bacteria to terramycin closely parallels that to aureomycin.

It has been emphasized[11] that many factors influence the results of antibiotic sensitivity tests, e.g., the method employed, composition of the medium used, size of the inoculum, temperature and duration of incubation, and the choice of complete vs. partial inhibition as the end point. The characteristic

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instability of aureomycin during prolonged incubation at 37° C. presents an additional complication. However, any yearly variations in sensitivity of any bacterial species found with this drug cannot be ascribed to this property, since all strains tested against aureomycin were subjected to the same effect. Throughout the period surveyed no technical innovations were introduced, and all elements in the performance of the test were kept constant. Thus any reported sensitivity changes cannot be ascribed to technical variations.

METHODS

The tube dilution method is employed exclusively in this laboratory for determining antibiotic sensitivity. The type of medium, preparation of antibiotic standards, size of inoculum for each species, temperature, and duration of incubation are all carefully controlled. Freshly prepared meat extract broth, pH 7.0 to 7.2, is used as the medium and diluent. Concentrated stock antibiotic standard solutions are prepared by dissolving standard aureomycin and chloramphenicol powder* in distilled water to a final concentration of 2.0 mg. per milliliter. The stock solutions are prepared each month and divided among a number of small sterile test tubes. These are stored in the freezing compartment of the refrigerator. Just prior to the determination a series of dilutions covering the range of concentrations necessary to perform the assays are prepared from one individual stock tube, thereby avoiding the deleterious effect of repeated thawing and refreezing of the stock solution. The used stock tube and dilute solutions are always discarded and never kept for future use. As an additional check against possible deterioration of the stock antibiotic solutions, a simultaneous test is always performed using a fixed inoculum of microorganisms routinely employed as a standard for each antibiotic; Bacillus No. 5, a strain of *Bacillus cereus* with aureomycin and *K. pneumoniae*, type A, MSH No. 067, with chloramphenicol. These are regularly inhibited by 0.2 μg per milliliter of aureomycin and 0.5 μg per milliliter of chloramphenicol. On rare occasions when amounts greater than those were found necessary to inhibit the growth of these bacteria indicating deterioration of the stock antibiotic solutions, the tests were repeated with freshly prepared stock solutions. Because of differences in growth potential it was necessary to vary the size of the inoculum with some species, but the inoculum size for any given species was kept constant during the entire period under investigation. Ordinarily, except as noted below, plain broth is inoculated with the organism under test and incubated for six hours at 37° C. and then diluted 1:1,000,000. For *Streptococcus pyogenes* and *Pneumococcus*, 1 per cent glucose broth is substituted for plain broth during preliminary incubation, and the six-hour culture is diluted only 1:10,000. With *H. influenzae* the initial incubation period is twenty-four hours and the final dilution is 1:100. Although different-sized inocula might have produced end points different from those to be reported, in order to achieve a valid basis for comparison over the years it was believed necessary to select and maintain a uniform inoculum for each species of bacteria. The assays are ordinarily performed in 2 ml. amounts, but where blood is necessary to insure growth of the microorganism the total volume in each tube is increased to 5 ml. and sufficient washed sterile sheep cells are added to each tube to make a final 2 per cent concentration.

The test is performed by adding one milliliter of the standardized inoculum to a series of tubes containing varying concentrations of the antibiotic. The end point is read after eighteen hours' incubation at 37° C. This is the first clear tube, indicating complete visual inhibition of growth, containing the least amount of drug. A growth control tube containing the test inoculum without antibiotic is employed with each determination.

RESULTS

The data have been divided into three periods corresponding to the years when the organisms were isolated and the tests performed. To cover the broad

*Kindly supplied by Lederle Laboratories Division, New York, N. Y., and Parke, Davis & Company, Detroit, Mich., respectively.

TABLE I. YEARLY COMPARISON OF SENSITIVITY OF VARIOUS MICROORGANISMS TO AUREOMYCIN

ORGANISM	YR.	MINIMAL INHIBITORY CONCENTRATION	SENSITIVE				RESISTANT				MARKEDLY RESISTANT	
			μG OR LESS/ML.		2-4 μG/ML.		5-10 μG/ML.		11-100 μG/ML.		>100 μG/ML.	
		TOTAL STRAINS	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
Esch. coli	'49	99	8	8.0	57	58.0	25	25.0	6	6.0	3	3.0
	'50	108	8	7.0	45	42.0	37	34.0	16	15.0	2	2.0
	'51	64	7	11.0	23	36.0	15	23.5	10	15.5	9	14.0
Pseudomonas aeruginosa	'49	54	0	-	1	2.0	16	29.5	36	66.5	1	2.0
	'50	50	0	-	0	-	15	30.0	32	64.0	3	6.0
	'51	28	0	-	2	7.0	6	21.5	20	71.5	0	-
Proteus	'49	35	1	3.0	2	6.0	6	17.0	14	40.0	12	34.0
	'50	54	0	-	2	4.0	4	7.0	15	28.0	33	61.0
	'51	36	2	5.5	0	-	1	3.0	11	30.5	22	61.0
Aerobacter aerogenes	'49	36	5	14.0	21	58.0	7	19.5	3	8.5	0	-
	'50	42	2	5.0	17	40.5	12	28.5	8	19.0	3	7.0
	'51	4	0	-	3	75.0	1	25.0	0	-	0	-
Salmonella (including E. typhosa)	'49	12	1	8.5	9	75.0	2	16.5	0	-	0	-
	'50	2	0	-	1	50.0	0	-	1	50.0	0	-
	'51	7	2	28.5	2	28.5	2	28.5	1	14.5	0	-
K. pneumoniae	'49	5	3	60.0	2	40.0	0	-	0	-	0	-
	'50	6	3	50.0	1	16.7	2	33.3	0	-	0	-
	'51	9	5	55.5	4	44.5	0	-	0	-	0	-
Alcaligenes fecalis	'49	4	0	-	2	50.0	2	50.0	0	-	0	-
	'50	11	6	55.0	1	9.0	1	9.0	2	18.0	1	9.0
	'51	3	0	-	2	66.6	1	33.3	0	-	0	-

TABLE I—CONT'D

ORGANISM	YR.	MINIMAL INHIBITORY CONCENTRATION TOTAL STRAINS	SENSITIVE				RESISTANT				MARKEDLY RESISTANT	
			μG OR LESS/ML.		2-4 μG/ML.		5-10 μG/ML.		11-100 μG/ML.		>100 μG/ML.	
			NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
H. influenzae	'49	1	1	100.0	0	–	0	–	0	–	0	–
	'50	17	13	76.0	2	12.0	2	12.0	0	–	0	–
	'51	1	1	100.0	0	–	0	–	0	–	0	–
Staph. aureus	'49	59	56	95.0	3	5.0	0	–	0	–	0	–
	'50	91	79	86.5	8	9.0	4	4.5	0	–	0	–
	'51	86	67	78.0	9	10.5	3	3.5	7	8.0	0	–
Staph. albus	'49	17	15	88.0	2	12.0	0	–	0	–	0	–
	'50	28	23	82.5	1	3.5	2	7.0	2	7.0	0	–
	'51	16	10	62.5	2	12.5	0	–	4	25.0	0	–
Str. fecalis	'49	34	15	44.0	16	47.0	1	3.0	1	3.0	1	3.0
	'50	73	28	38.5	8	11.0	4	5.5	22	30.0	11	15.0
	'51	57	20	35.0	8	14.0	2	3.5	16	28.0	11	19.5
Str. viridans	'49	30	21	70.0	9	30.0	0	–	0	–	0	–
	'50	41	35	85.0	5	12.5	1	2.5	0	–	0	–
	'51	36	29	80.5	5	14.0	2	5.5	0	–	0	–
Str. pyogenes	'49	14	13	93.0	0	–	1	7.0	0	–	0	–
	'50	34	34	100.0	0	–	0	–	0	–	0	–
	'51	19	15	79.0	4	21.0	0	–	0	–	0	–
Pneumococcus	'49	11	11	100.0	0	–	0	–	0	–	0	–
	'50	11	11	100.0	0	–	0	–	0	–	0	–
	'51	10	10	100.0	0	–	0	–	0	–	0	–

TABLE II. YEARLY COMPARISON OF SENSITIVITY OF VARIOUS MICROORGANISMS TO CHLORAMPHENICOL

ORGANISM	YR.	MINIMAL INHIBITORY CONCENTRATION	SENSITIVE				RESISTANT				MARKEDLY RESISTANT	
			2 μG OR LESS/ML.		3-6 μG/ML.		7-10 μG/ML.		11-100 μG/ML.		> 100 μG/ML.	
		TOTAL STRAINS	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
Esch. coli	'49	33	10	30.0	18	55.0	2	6.0	2	6.0	1	3.0
	'50	114	56	49.0	40	35.0	7	6.0	7	6.0	4	4.0
	'51	90	22	24.0	43	48.0	5	5.5	12	13.5	8	9.0
Pseudomonas aeruginosa	'49	33	0	–	4	12.0	4	12.0	23	70.0	2	6.0
	'50	53	0	–	3	5.5	14	26.5	29	55.0	7	13.0
	'51	54	0	–	1	2.0	17	31.5	28	51.5	8	15.0
Proteus	'49	18	0	–	6	33.3	8	44.5	4	22.2	0	–
	'50	53	2	4.0	24	45.0	7	13.0	18	34.0	2	4.0
	'51	72	3	4.0	25	35.0	12	16.5	24	33.3	8	11.2
Aerobacter aerogenes	'49	8	4	50.0	2	25.0	1	12.5	1	12.5	0	–
	'50	41	19	47.0	12	29.0	3	7.0	6	14.5	1	2.5
	'51	8	5	62.5	1	12.5	0	–	2	25.0	0	–
Salmonella (including E. typhosa)	'49	10	6	60.0	4	40.0	0	–	0	–	0	–
	'50	2	2	100.0	0	–	0	–	0	–	0	–
	'51	7	5	72.0	1	14.0	1	14.0	0	–	0	–
K. pneumoniae	'49	–	–	–	–	–	–	–	–	–	–	–
	'50	6	5	83.3	1	16.7	0	–	0	–	0	–
	'51	12	9	75.0	2	16.7	1	8.3	0	–	0	–
Alcaligenes fecalis	'49	2	0	–	0	–	0	–	2	100.0	0	–
	'50	11	4	36.5	2	18.0	1	9.0	4	36.5	0	–
	'51	3	1	33.3	1	33.3	1	33.3	0	–	0	–

TABLE II—CONT'D

ORGANISM	YR.	MINIMAL INHIBITORY CONCENTRATION	SENSITIVE				RESISTANT				MARKEDLY RESISTANT	
			2 μG OR LESS/ML.		3-6 μG/ML.		7-10 μG/ML.		11-100 μG/ML.		>100 μG/ML.	
		TOTAL STRAINS	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
H. influenzae	'49	–	–	–	–	–	–	–	–	–	–	–
	'50	16	14	87.5	2	12.5	0	–	0	–	0	–
	'51	1	1	100.0	0	–	0	–	0	–	0	–
Staph. aureus	'49	18	3	16.7	15	83.3	0	–	0	–	0	–
	'50	34	26	76.5	6	17.5	2	6.0	0	–	0	–
	'51	43	21	49.0	19	44.0	0	–	3	7.0	0	–
Staph. Albus	'49	2	0	–	2	100.0	0	–	0	–	0	–
	'50	12	7	58.0	1	8.5	1	8.5	3	25.0	0	–
	'51	6	5	83.3	0	–	0	–	1	16.7	0	–
Str. fecalis	'49	19	8	42.0	9	47.5	2	10.5	0	–	0	–
	'50	72	39	54.0	28	39.0	1	1.5	4	5.5	0	–
	'51	80	41	51.0	29	36.0	5	6.5	5	6.5	0	–
Str. viridans	'49	8	4	50.0	4	50.0	0	–	0	–	0	–
	'50	3	2	66.6	1	33.3	0	–	0	–	0	–
	'51	20	14	70.0	5	25.0	1	5.0	0	–	0	–
Str. pyogenes	'49	1	0	–	1	100.0	0	–	0	–	0	–
	'50	5	5	100.0	0	–	0	–	0	–	0	–
	'51	7	5	71.5	2	28.5	0	–	0	–	0	–
Pneumococcus	'49	2	1	50.0	1	50.0	0	–	0	–	0	–
	'50	3	3	100.0	0	–	0	–	0	–	0	–
	'51	2	1	50.0	1	50.0	0	–	0	–	0	–

range of values, the results of the sensitivity tests are listed in five categories according to the minimal concentration of drug found necessary to inhibit the growth of each organism. These are shown in Tables I and II. Attention is directed to the fact that the same categories of concentrations are not employed with both antibiotics as it has been noted[12] that a greater amount of chloramphenicol than aureomycin on a weight basis, is usually required to inhibit the same susceptible strain of bacteria in vitro. In addition there is no intent on the part of the author to make any direct comparisons with respect to the lack

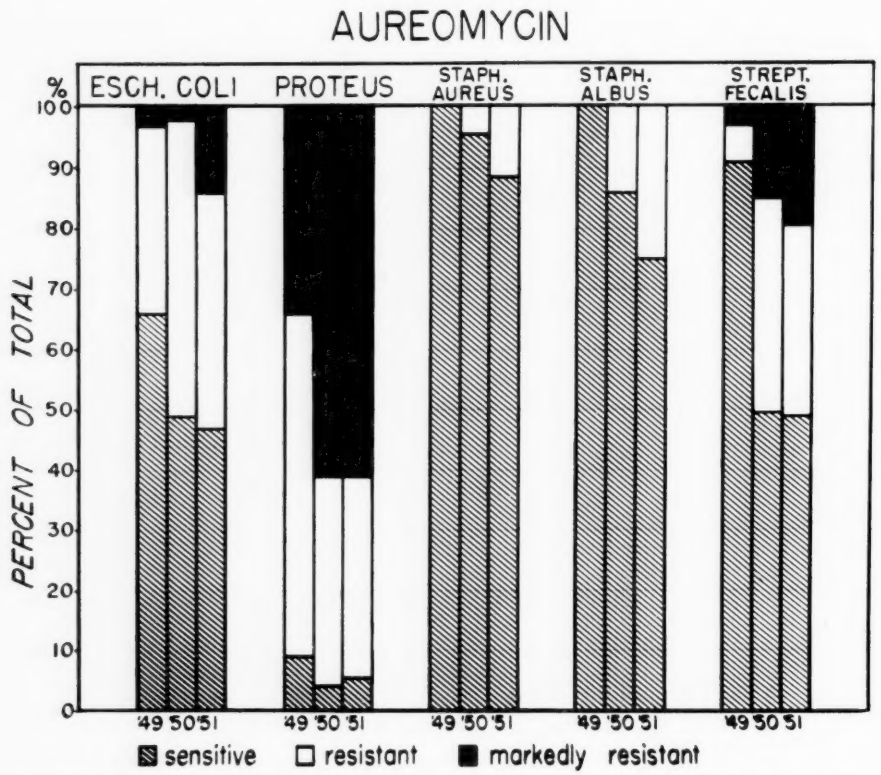

Fig. 1.—Development of increased resistance to aureomycin of *Esch. coli, Proteus, Staph. aureus, Staph. albus,* and *Str. fecalis* in the course of the past three years. Strains inhibited by 4 μg per milliliter or less classified as Sensitive, 5 to 100 μg per milliliter as Resistant, and those not inhibited by 100 μg per milliliter as Markedly Resistant.

of development of bacterial resistance between the two antibacterial agents discussed but merely to record the values observed for any one drug and any one species at different years.

Based upon clinical impression and in order to facilitate the analysis of these data, the strains were arbitrarily designated as Sensitive, Resistant and Markedly Resistant according to the concentration of antibiotic necessary for bacterial inhibition. Those microorganisms inhibited by 4 μg per milliliter or less of aureomycin were classified as "Sensitive," 5 to 100 μg per milliliter as "Resistant," and those not inhibited by 100 μg per milliliter as "Markedly

Resistant.'' With respect to chloramphenicol, those inhibited by 6 μg per milliliter or less were considered ''Sensitive,'' 7 to 100 μg per milliliter as ''Resistant,'' and those requiring more than 100 μg per milliliter as ''Markedly Resistant.''

As may be noted, the sensitivity pattern of a number of groups of bacteria to both antibiotics has altered with the passage of time. These changes are in the direction of increasing resistance, as indicated by a relative decline in the percentage of Sensitive strains with a corresponding increase in the proportion

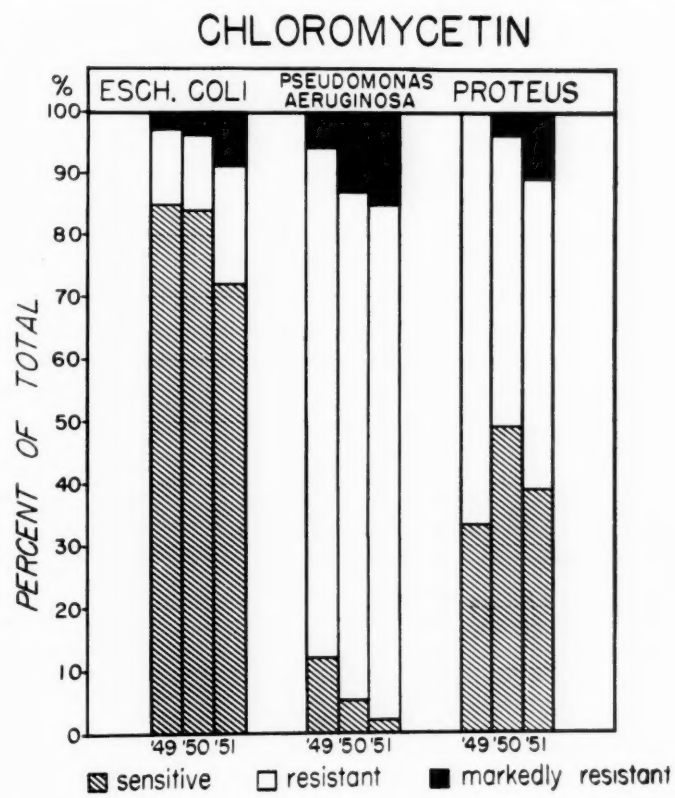

Fig. 2.—Development of increased resistance to chloramphenicol of *Esch. coli, Ps. aeruginosa,* and *Proteus* in the course of the past three years. Strains inhibited by 6 μg per milliliter or less are classified as Sensitive, 7 to 100 μg per milliliter as Resistant, and those requiring more than 100 μg per milliliter as Markedly Resistant.

of Resistant or Markedly Resistant strains. Those involved are *Esch. coli, Proteus, Staph. aureus, Staph. albus,* and *Str. fecalis* to aureomycin and *Esch. coli, Ps. aeruginosa,* and *Proteus* to chloramphenicol. The susceptibility status of the other species listed has remained more or less constant during the period studied, or too few strains have been isolated to draw any definite conclusions. *H. influenzae, Str. viridans, Str. pyogenes,* and *Pneumococcus* have been found to be highly sensitive to the action of both antibiotics, and unusually resistant strains have not been discovered to date. The situation with respect to the

staphylococci is of particular interest in view of the previously described experience with penicillin. It may be seen from the tables that during the first year of their use, all strains of staphylococci proved sensitive to both aureomycin and chloramphenicol, but in the next two years the percentage of resistant forms has increased progressively.

The data concerned with those groups demonstrating increasing bacterial resistance over the years were subjected to statistical analysis by means of the chi square test.* A probability value less than 0.05 was considered as significant. With respect to aureomycin, the changes observed with *Esch. coli* and *Str. fecalis* were found to be highly significant (0.0015, and less than 0.0001), *Proteus* and *Staph. aureus* significant (0.022 and 0.021), and *Staph. albus* of questionable significance (0.078). When the figures, which seemed to indicate a progressive increase in resistance of *Esch. coli, Ps. aeruginosa,* and *Proteus* to chloramphenicol were submitted to the same analysis, the results did not prove to be significant. In this instance the probability values were computed as 0.199, 0.42, and 0.140, respectively. Thus, with chloramphenicol, conclusions based upon the data herein reported must be held in abeyance, even though at first glance they appear to be highly suggestive.

The increased bacterial resistance to aureomycin and chloramphenicol acquired by several species of bacteria during the past three years, as described above, is depicted graphically in Figs. 1 and 2. These changes are expressed by a progressive diminution in the ratio of the percentage of Sensitive strains to the Resistant or Markedly Resistant strains of each species.

SUMMARY AND CONCLUSIONS

In the light of the experience with older chemotherapeutic agents, a survey of the sensitivity to aureomycin and chloramphenicol of a number of different bacteria isolated from pathologic material was undertaken to determine whether any increase in bacterial resistance to either drug has developed within any particular species since the introduction of these newer antibiotics approximately three years ago. Gradual emergence of this complication has been found with *Esch. coli, Proteus, Staph. aureus, Staph. albus,* and *Str. fecalis* with aureomycin and *Esch. coli, Ps. aeruginosa,* and *Proteus* with chloramphenicol. When the data concerned with these groups were subjected to statistical analysis by means of the chi square test, only those figures indicating increasing bacterial resistance of *Esch. coli, Proteus, Staph. aureus* and *Str. fecalis* to aureomycin were found to be significant. The actual values quoted are not considered as indicating relative superiority of one drug over another with respect to the lack of development of bacterial resistance, since the existence of so many clinical and technical variables precludes justifiable comparison. The figures merely represent values observed for any one drug and any one species at different years.

The marked variation in susceptibility to these antibiotics shown by individual members of some groups strongly emphasizes the necessity for performing sensitivity tests in order to select the most effective antimicrobial agent against these microorganisms.

*I am greatly indebted to Mrs. Constance Percy for the statistical analysis.

Whether continued and even more widespread clinical use of these antibiotics will further enhance this trend remains to be seen. The possibility that resistant strains may also develop at a later date in some of the species in which no alterations have been as yet detected must also be entertained. The developments herein described have very important implications since it portends a further constriction of the available therapeutic armamentarium against a number of infectious agents capable of producing serious disease.

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THE AVIDITY OF TEST TOXINS IN RELATIONSHIP TO TETANUS ANTITOXIN TITRATIONS

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INTRODUCTION

SOME interesting and unexpected findings have resulted from attempts in different laboratories to assay tetanus antitoxins accurately. These assays are done in the following way:

One-tenth of a unit of standard antitoxin is mixed with varying amounts of toxin, allowed to stand for one hour at room temperature, and the mixtures injected into mice or guinea pigs. The smallest amount of toxin which will kill the animal in ninety-six hours under these conditions is the test dose. The test dose is then mixed with varying amounts of the unknown antitoxin and injected into other animals. The amount of antitoxin in such a mixture which kills in ninety-six hours then represents one-tenth of a unit, and from this the number of units per milliliter in the unknown can be calculated. In the final titration the standard and the unknown are titrated simultaneously so that the reagents and conditions will be the same for both antitoxins.

In this test the comparison is between the unknown and the standard antitoxin, while the toxin is only the intermediary. It would, therefore, seem that the quality and strength of the toxin would be immaterial if suitably diluted to obtain an end point. Strangely enough, the unit value of an antitoxin may vary according to the toxin used, even though the same standard antitoxin is used throughout the titration.

This observation is interesting from the academic standpoint, and is of practical significance in the titration of prophylactic and therapeutic serums.

This peculiarity of tetanus antitoxin titrations has been recognized for some time. In August, 1934, the Commission on Biological Standardization of the Health Organization of the League of Nations decided to study the problem.[1-5] One of the conclusions reached in this study was that "the accuracy of the assay of a given tetanus antitoxin is greatly decreased when different toxins are used for the titration." Though as a rule toxins from different sources give divergent titers, Ipsen[6] showed that when the strain of organism, length of incubation, and medium were the same, the toxins produced by several laboratories were qualitatively the same. Lewellyn Smith[7] did not find any evidence indicating the presence of multiple antigens in toxins, basing this conclusion on titrations of sera prepared in rabbits immunized against various toxins. On the other hand Petrie,[8] in a very thorough study of the problem, concluded that crude toxins are a mixture of primary toxin molecules and a

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secondary variant. These vary in antigenicity, and as a result antitoxins contain at least two antigenic components. Lewellyn Smith[3] quoted an unpublished work of Hartley stating that toxin-antitoxin mixtures dissociate on dilution, the degree of dissociation varying with the sample of toxin. In 1942[7] she recorded differences in dissociation when different toxins were diluted. She did not, however, directly connect these differences with the differences in titer of an antitoxin when different toxins were used in the assay. Barr[9] mentions the relative difference in the amount of antitoxin in strong and dilute neutral toxin-antitoxin mixtures. These differences will be used here to "correct" for the errors which we believe are due to variations in dissociation.

First we will show that the differences in antitoxin titers with different toxins are real and reproducible. Next we will point out the range of these differences with evidence that the difference between toxins is correlated with denaturation. Finally, a method will be presented for correction of the differences as found.

EXPERIMENTAL

The study was originally started with two toxins: the old U. S. control toxin H4 and the new toxin N designed to replace it. It was found that many antitoxins when titrated with toxin N gave a lower potency than when titrated with toxin H4. It was also found that the flocculating antitoxin in use, Lot A, varied more in this respect than many other antitoxins. It was therefore decided to confine the study to comparisons between antitoxin A and the U. S. standard tetanus antitoxin, thus fewer animals would be required to obtain a significant result. All titrations were done with Swiss mice (National Institutes of Health strain) using 1 ml. of the toxin-antitoxin mixture subcutaneously. The mixtures were allowed to react for one hour at room temperature before the injection, and the animals were held for five to eight days before being discarded. Two mice were used for dilution, and approximately 20 per cent increments of antitoxin were used. For confirmatory titrations three or more mice were used with approximately 10 per cent increments.

TABLE I. PROTOCOL ILLUSTRATING THE DIFFERENCE IN TITER OF ANTITOXIN A TITRATED WITH TOXINS H4 AND N AT THE 0.2 UNIT LEVEL

TOXIN	ANTITOXIN	BUFFERED SALINE pH 7.0	H4 TOXIN 4.3 MG. PER ML.	N TOXIN 16.6 MG. PER ML.	
0.2 ml.	0.16 ml.	0.64 ml.	24 24	24 24	
0.2 ml.	0.20	0.60	24 24	96 96	N.I.H. Standard
0.2 ml.	0.24	0.56	S S	S S	Antitoxin contains
0.2 ml.	0.28	0.52	S S	S S	5 units per ml. It
0.2 ml.	0.32	0.48	S S	S S	was diluted 1/5
0.2 ml.	0.16	0.64	20 20	20 20	Antitoxin A diluted
0.2 ml.	0.20	0.60	24 26	20 20	1/136
0.2 ml.	0.24	0.56	96 96	24 24	
0.2 ml.	0.28	0.52	S S	48 48	
	0.32	0.48	S S	96 96	
			Titer of A 113 units	Titer of A 85 units	

1 ml. of mixture given to each mouse subcutaneously.
Figures under toxins represent hours of survival.
S indicates survival for more than five days.

An illustrative protocol, Table I, indicates the general arrangement of such an experiment and shows the difference in titer of antitoxin A when two different toxins were used.

Differences in Titre of Antitoxin A When Titrated With Two Toxins.—To show that the differences shown in Table I are real and reproducible, this test was repeated twelve times using toxins H3 and N. The average value of antitoxin A when titrated with toxin H3 was found to be 114 units per milliliter. The average value with toxin N was 78.4 units per milliliter. The values with toxin H3 ranged from 106 to 119 units per ml., with toxin N from 68 to 85 units per milliliter. Thus in all tests the value of antitoxin A with H3 was greater than with N.

Effect of Age and Chemical Treatment on Toxins.—Antitoxin A has been titrated against the standard antitoxin using a number of test toxins. The titers obtained are shown in Table II along with some data on the source of the toxins.

TABLE II. ASSAY OF ANTITOXIN A USING A NUMBER OF TEST TOXINS

TOXIN	UNIT VALUE OF A	SOURCE
X	68	Details of production unknown. Dried.
Crystallized	75.3	Highly purified. Received from Dr. Pillemer.
136 liquid	77.3	Made from horse meat infusion broth with glucose. Inoculated with strain Tulloch II. Liquid.
136	85.3	Same as above but precipitated with ammonium sulfate and dried.
N	85	Pool of several toxins prepared with Tulloch II and precipitated with ammonium sulfate and dried. Present control toxin.
1722M	85	Details of production unknown.
137 liquid	85.3	Made from Hall Medium. Inoculated with Tulloch II.
137	85.3	Above but precipitated with ammonium sulfate and dried.
132	85.3	Muellers Medium. Tulloch II. Precipitated and dried.
M	85.7	Horse meat infusion broth. Tulloch II precipitated and dried.
1748	85.5	Preserved with phenol mercuric acetate. Jan., 1949.
C35	85 91.5	Received from Dr. Miles, Hampstead, England. (Duplicate titrations.)
1748C	99	Same as 1748 but chloroform added to saturation and incubated at 35° C. for 8 days.
1754M	87.3	Preserved with 0.5% phenol. March, 1949.
151	85.3	Hall Medium Tulloch II precipitated and dried.
151C	96.5	Same as above but chloroform added before precipitation.
35626	80	
35626C	101.6	Above but incubated 2 days at 37° C. with chloroform.
H4	116	Old control toxin. Beef infusion broth. Tulloch II and III. Precipitated with ammonium sulfate and dried. Prepared 1926-27.
H3	112	Same as H4 but filled into tubes at a different time.
81475	85.7	Prepared Oct., 1948, Strain 47 from Dr. Mueller. Hog stomach autolysate medium. Chloroform preservative.
81475 old	119	Same as above but after standing in refrigerator with frequent removal for tests.
p88	163	Above toxin after precipitation with ammonium sulfate and drying.

This table illustrates the differences in antitoxin unit values when one antitoxin is titrated with various toxins. Values as low as 68 and as high as 163 were obtained. On the other hand, most of the toxins gave a value of about 85 units per milliliter. Toxins produced from various media, with various strains, and from various sources often give essentially the same titer.

The toxins which give antitoxin A a high titer, H4, H3, 81475 old, p88, 1748C, 35626C and 151C are either very old or have been denatured by treatment with chloroform. In addition, all of these have a certain amount of cloudiness which may be evidence of denaturation. All but one of the toxins giving a low titer are quite clear. Toxin 81475 when received was quite clear and gave a value of 85 units. On ageing it became turbid and gave a value of 119 units. These figures indicate that freshly prepared undenatured tetanus toxins are fairly uniform in quality.

Dissociation of Toxin-Antitoxin Mixtures as a Cause of Variation in Antitoxin Titers.—The two values obtained in Table I may be explained on the basis of dissociation. We assume that the combination of standard antitoxin and H4 toxin is complete. The same assumption is made for standard antitoxin and toxin N. Antitoxin A and toxin H4 combine moderately, and antitoxin A and toxin N combine weakly. Antitoxin A and toxin H4 would dissociate leaving free toxin which would kill the mice unless more antitoxin were added. In the Table 0.24 ml. of antitoxin is needed for neutrality with antitoxin A and toxin H4 and only 0.20 with standard antitoxin and H4 toxin.

Antitoxin A and toxin N combine even less firmly and even more antitoxin is needed, 0.32 ml. vs. 0.20 ml. By calculating the end points without considering dissociation these figures give us the two values of 113 and 85 units.

Actually, there would probably be some dissociation in all of the mixtures, but if there were different amounts of dissociation in the various combinations the theory would apply. It is not necessary to postulate a very low avidity between toxin N and antitoxin A. An especially strong avidity between toxin N and standard antitoxin would cause the same effect.

Correction of Errors Due to Dissociation by Means of Titration of Dilute Mixtures.—If the above assumptions are correct one would expect that the differences in the titers of an antitoxin when two toxins are used would be increased when the mixtures are diluted. This follows from the premise that dissociation is greater in dilute solutions. Actual experiments do show greater differences when dilute mixtures are used.

One might conclude that the concentration of the reagents might be increased to such an extent that the two antitoxin titers would be equal. Experiments indicate that this is not practical. We have, therefore, made duplicate titrations, one with dilute reagents and one with reagents at the usual strength. These results suitably treated mathematically give us essentially the same antitoxin titers with the two toxins. In order to understand the mathematical treatment of the results, some discussion of the effect of dilution of toxin-antitoxin mixtures is desirable.

The combination of any toxin and antitoxin is probably never absolutely complete. The dissociation will depend upon the inherent natures of the toxin and antitoxin and their concentration. The greater the dilution the greater will be the dissociation. The analogy would be that of a salt of a weak acid and a weak base in which ionization is greater in dilute than in concentrated solutions. If, then, a neutralized (nontoxic) mixture of toxin and antitoxin is

TABLE III. TITRATION OF ANTITOXIN A WITH TWO TOXINS, USING TWO CONCENTRATIONS AND SHOWING VALUES AS FOUND AND ADJUSTED

1	2	3	4		5		6		7		8		9		10		11		12
			TOXIN X 2.5 MG. PER ML.								TOXIN 1748C DILUTED 1/14								
TOXIN (ML.)	ANTI-TOXIN (ML.)	PHOSPHATE BUFFER pH 7.6 (ML.)	STANDARD ANTITOXIN		A ANTITOXIN		STANDARD ANTITOXIN DILUTE		A ANTITOXIN DILUTE		STANDARD ANTITOXIN		A ANTITOXIN		STANDARD ANTITOXIN DILUTE		A ANTITOXIN DILUTE		DIFFERENCE
1.2	0.32	1.48													3	4	4	4	
1.2	0.36	1.44													4	6	4	S	
1.2	0.40	1.40											2	2	4	4	S	S	
1.2	0.44	1.36	2	2*	1	2	2	3	2	2	2	2	2	2	5	5	S	S	
1.2	0.48	1.32	3	3	1	2	2	2	2	2	2	2	2	S	5	6	S	S	
1.2	0.52	1.28	4	4	2	3	3	3	2	2	2	3	3	3	S	S	S	S	
1.2	0.56	1.24	6	S	3	3	4	6	2	3	2	2	4	4	S	S	S	S	
1.2	0.60	1.20	S	S	4	4	S	S	3	3	3	4	S	S	S	S	S	S	
1.2	0.64	1.16	S	S	6	S	S	S	6	6	3	4	S	S	S	S	S	S	
1.2	0.68	1.12	S	S	S	S	S	S	S	S	4	6	S	S	S	S	S	S	
1.2	0.72	1.08	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
			$\frac{170 \times 5 \times 0.54}{10 \times 0.62} = 74$ units per ml.								$\frac{170 \times 5 \times 0.68}{10 \quad 0.56} = 103$ units per ml.								29 units
			$\frac{170 \times 5}{10}\left[\frac{2 \times 0.54}{0.62} - \left(\frac{0.56}{0.64}\right)^{1.5}\right] = 79$ units per ml. (adjusted)								$\frac{170 \times 5}{10}\left[\frac{2 \times 0.68}{0.56} - \left(\frac{0.48}{0.34}\right)^{1.5}\right] = 63.7$ units per ml. (adjusted)								15.3 units

Each mouse receives 1 ml. of the mixture subcutaneously. The standard antitoxin contains 5 units per milliliter and is diluted 1/10. Antitoxin A is diluted 1/170. Immediately after mixing toxin-antitoxin and buffer, 0.5 ml. of the mixture is added to 4.5 ml. of buffer. This is the dilute mixture. All are allowed to stand for one hour at room temperature before inoculating. The end points in columns 4, 5, and 11 are obtained by interpolation.

*Figures represent days of survival of each of two mice. S represents survival for more than seven days.

diluted there will be an increase in the amount of toxin dissociated. This amount of free toxin would kill the animals. More antitoxin would then be necessary to keep the mixture neutral or nontoxic. *The more dilute solutions would then require proportionately more antitoxin than the more concentrated solutions.*

The above is quite obvious and in an experiment we would expect to invariably find relatively more antitoxin needed in dilute mixtures. Due to another effect of dilution, to be mentioned below, this is not always so.

A mixture which contains the amount of antitoxin which will neutralize one M.L.D. of pure toxin plus two M.L.D. of pure toxin will kill mice. The ratio of toxin to antitoxin will be 2:1. Enough antitoxin to neutralize 10 M.L.D. of pure toxin plus 11 M.L.D. of toxin will also kill mice in about the same time. In this case the ratio is 11:10. It is apparent that *in more dilute solutions,* containing less of the reagents, *relatively less antitoxin in proportion to toxin is needed* to save the animals.

The extent of this effect will be influenced by the strength of the crude toxin used. The number of M.L.D. per test dose of antitoxin is a measure of the strength of the toxin and is an indicator of the proportion of toxin to toxoid.

Toxin x in Table III requires slightly *more* antitoxin in the dilute mixture. This is reasonable in view of the discussion on dissociation. With toxin 1748C we find that *less* antitoxin is required in dilute solutions. This is accounted for by the above-described process. Both effects are applicable to both toxins, however, toxin 1748C is weak* making the dilution effect more pronounced with this toxin, thus obscuring the dissociation effect.

With the above in mind, an attempt was made to set up a formula to adjust for the effects of dissociation. It was set up so as to cancel out the irregularities of the dilution effect mentioned above. Titrations were set up at the usual strength and at a 1/10 dilution. The following formula was then applied:

$$\text{Adjusted unitage} = \frac{t\ U}{s}\left[\frac{2S}{T} - \left(\frac{SD}{TD}\right)^{k}\right]$$

Where U = No. units per milliliter in standard antitoxin
t = Denominator of dilution of test antitoxin
s = Denominator of dilution of standard antitoxin
T = Volume of test antitoxin necessary
S = Volume of standard antitoxin necessary
TD = Volume test antitoxin necessary when diluted 1/10
SD = Volume of standard antitoxin necessary when diluted 1/10
k = constant = 1.5

Such a titration is illustrated in Table III. The antitoxin values with the two toxins have been obtained using the undiluted mixtures. This is the customary method. Using both diluted and undiluted mixtures the above formula had been applied. The adjusted titers with the two toxins coincide more closely than the original titers.

*The ratio of the test dose volume to the M.L.D. volume of toxin 1748, the parent toxin of 1748C, is only 80, while this ratio for toxin X is 2630.

In an empiric formula such as this the only "proofs" of its validity are the results obtained. In Table IV are the adjusted and unadjusted values of antitoxin A when tested repeatedly with a number of toxins.

TABLE IV. VALUES OF ANTITOXIN A WITH A NUMBER OF TOXINS AS FOUND AND ADJUSTED

TOXIN	VOLUME OF ANTITOXIN IN ML. AT END POINT				NO. UNITS FOUND	ADJUSTED UNITAGE
	A DIL.	A	S	S DIL.		
X	0.64	0.62	0.54	0.56	74.0	78.2
X	0.66	0.60	0.52	0.56	73.7	80.7
X	0.80	0.74	0.58	0.58	66.6	80.7
X	0.68	0.66	0.64	0.62	82.4	83.7
Ave.					74.2	80.8
35626	0.56	0.64	0.60	0.56	79.7	74.0
35626	0.70	0.74	0.70	0.67	80.4	79.2
Ave.					80.0	76.6
35626C	0.52	0.66	0.76	0.64	97.9	79.9
35626C	0.52	0.68	0.80	0.68	100.0	72.2
35626C	0.52	0.64	0.78	0.62	103.5	98.6
35626C	0.52	0.68	0.84	0.64	105.0	93.5
Ave.					101.6	86.0
N	0.58	0.60	0.54	0.52	76.5	83.3
N	0.58	0.58	0.56	0.52	82.1	90.1
N	0.63	0.60	0.56	0.55	79.3	89.2
N Ave.					79.3	87.5
1748C	0.34	0.56	0.68	0.48	103.0	63.8
1748C	0.36	0.64	0.68	0.44	90.3	65.4
1748C	0.46	0.72	0.74	0.56	87.3	61.2
1748C	0.54	0.76	0.72	0.54	80.5	75.6
1748C	0.54	0.76	0.78	0.57	87.2	82.4
1748C Ave.					89.6	69.7
H4	0.60	0.56	0.74	0.82	112	79.9
H4	0.54	0.54	0.74	0.81	119	76.3
H4 Ave.					115	78.9

The adjusted values using various toxins approach each other more closely than do the unadjusted figures. Analysis of variance reveals that the unadjusted values are significantly different from each other, while the same cannot be said for the adjusted values. The differences between the adjusted and unadjusted values are significant for some of the toxins.

There are several possible reasons why some of the adjusted values do not check too well. The earlier tests were concluded in four to six days. Later it was found that a number of animals died up to the eighth day, especially in the dilute mixtures. If these deaths were counted the results might be more uniform.

There is certain evidence that the curve of dissociation plotted against dilution is not linear. This suggests that if the tests were done on a different part of the curve, say 0.4 and 0.04 unit, rather than 0.1 and 0.01, the results may be improved.

DISCUSSION

There is clear-cut evidence that the titer of a given antitoxin is influenced by the toxin used in the titration.

These qualitative differences in toxins may be due to a denaturation process, for most freshly prepared toxins give a rather low titer and are usually clear. Older toxins and those which have been treated with chloroform (a protein denaturant) are often cloudy and give rise to higher titers. The cloudiness mentioned could be a manifestation of denaturation or at least some chemical or physical change.

It is quite possible that tetanus toxins at the moment of formation are qualitatively identical but from that time onward, a progressive deterioration or denaturation sets in, resulting eventually in a toxin of quite different characteristics. It is even possible that this deterioration begins during the incubation of the culture. This would account for the differences between the short growth and long growth toxins noted by Petrie.[8]

From the practical standpoint it is suggested that test toxins be handled in such a way as to prevent their deterioration as much as possible. The culture should be grown only a short time. No harmful preservatives should be used. It should be kept as cold as possible and should be preserved in the dried or frozen state. A possible index of the suitability of the toxin for titration might be its qualitative similarity to a fresh crystallized toxin.

The difference in titer of an antitoxin when different toxins are used may be explained by differences in binding power of toxin and antitoxin. This is expressed by the term avidity or by its opposite, dissociation. The concept is that an antitoxin may have a high avidity for one toxin and a lower avidity for another. The ratio of the two avidities for the same toxins may be different with another antitoxin. This, of course, is no fundamental explanation, as we do not know the nature of the forces producing avid and nonavid sera.

Divergent titration values of an antitoxin when tested with several toxins may be brought into closer agreement by the use of mathematical "corrections" based upon the dissociation of toxins and antitoxins. This furthers the argument that these variations are due to the differences in avidity.

SUMMARY

The titer of a given antitoxin may vary with the toxin used in the assay.

Most freshly prepared toxins, irrespective of methods of preparation, and one crystallized toxin gave similar titers with one antitoxin. Toxins which gave divergent titers were slightly turbid. These were very old or had been treated with chloroform. In one instance, a toxin which when fresh behaved normally, on ageing changed so as to give the test antitoxin a higher titer.

It has been shown that variation in titer could be due to variations in the dissociation of toxin-antitoxin complexes.

An empiric formula has been developed to adjust tetanus antitoxin titration values. When one antitoxin was titrated, application of this formula gave fairly consistent titers irrespective of the toxin used.

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THE STERILIZATION OF PENICILLIN AND STREPTOMYCIN BY ETHYLENE OXIDE

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THE number of applications to which ethylene oxide has been put has grown apace since its bactericidal activity was first emphasized[1-4] a few years ago. Besides its use in the sterilization of articles not susceptible to more common techniques,[5, 6] this chemical has been added to bacteriologic media which are thereby rendered sterile, but still support the growth of fastidious organisms.[7]

Observations made several years ago in our laboratories on the sterilization of antibiotics have been of interest to individuals concerned with this problem. Some commercial producers of penicillin are now routinely using ethylene oxide to sterilize their heat-labile products. Since simple, small-scale apparatus for applying this gas in a reasonably safe and convenient manner has been described[6] and is being produced for sale,* a summary of some of our tests on the sterilization of antibiotics may be of service to laboratories where this process may become necessary.

MATERIALS AND METHODS

1. *Treatment of Dry Antibiotics with Ethylene Oxide Vapor.—Preparation of samples:* The materials used were sodium penicillin-G,† and streptomycin, calcium chloride salt,‡ Solutions were prepared by adding distilled water to the unopened vials by a syringe. Aliquots were then dispensed into sterile serum bottles with a microburette, and the water was evaporated in a lyophilizing apparatus.

Contaminants: Three microorganisms were mixed with the lyophilized antibiotics: spores of *Aspergillus tamarii*, spores of *Bacillus globigii*, and cells of *Escherichia coli*. The first two were available as dried powders; the last as a paste obtained by centrifugation of a large volume of eighteen-hour broth culture.

Exposure to ethylene oxide vapor: The samples were placed in a vacuum desiccator connected with a mercury manometer, and the air was evacuated. Ethylene oxide vapor was admitted by connecting a bottle containing the cold liquid with the desiccator, and by allowing the liquid to evaporate until the desired pressure was reached. The pressure used in all the tests was 200 mm., which corresponds at 25° C. (the average room temperature) to a concentration of about 475 mg. of ethylene oxide per liter of space. When the desired time had elapsed, the desiccator was again evacuated, held under vacuum for one-half hour, and then air was admitted until atmospheric pressure was reached. The dry powder in each vial was taken up in 1 ml. sterile water, which was then diluted and assayed for sterility, antibiotic content, and toxicity.

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*American Sterilizer Co., Erie, Pa.
†Merck and Company, Inc., Rahway, N. J., lot No. 1280.
‡Merck and Company, Inc., Rahway, N. J., lot No. 1149.

Assay methods: Determinations of penicillin potency were made by a modification of the Vincent and Vincent method,[8] using *Bacillus subtilis* (09528) as the test organism. Dr. J. Forgacs kindly supervised the streptomycin assays done by the micromethod of Forgacs and Kucera.[9]

Sterility tests on penicillin: After exposure of penicillin-microorganism mixtures to ethylene oxide, sufficient Penicillinase-"S"* solution was added to some of the samples to neutralize the antibiotic present, so that an estimate of the number of viable microorganisms remaining in the mixture could be made. A control sample was included in each test to make certain that the penicillinase itself had no effect upon the organisms.

Determination of acute toxicity: (a) *Penicillin:* Serum bottles were filled with 30,000 units of dry penicillin by lyophilizing the appropriate amount of solution. Half of the samples were exposed to ethylene oxide vapor (475 mg. per liter) in the desiccator for six hours, followed by evacuation for thirty minutes. The remaining samples which served as controls were treated in the same manner, except that air was admitted instead of ethylene oxide. Each of ten of the exposed samples was taken up in one milliliter of sterile distilled water and injected subcutaneously at the dorsal part of the neck of a twenty gram male Swiss mouse. Ten controls were similarly injected. Five mice received one milliliter of sterile distilled water, and five were untreated.

(b) *Streptomycin:* The streptomycin toxicity tests were performed in the same way, with ten mice each receiving one milliliter containing 10,000 units of unexposed material, ten receiving an equal dosage of exposed sample, and five receiving distilled water.

2. *Treatment of Penicillin Solutions.*—One milliliter of a penicillin solution containing 1,000 units was placed in each of a number of vials, and contaminated grossly either with dry *B. globigii* spores or centrifuged *E. coli* cells. To the vials, held in a cold room at 4° C., 0.05, 0.10, or 0.20 ml. of cold liquid ethylene oxide were added by means of a chilled pipette. The mixtures stood at 4° C., and were assayed for penicillin and bacterial content by the procedures described above two and four hours after treatment began. Similar vials, to which no ethylene oxide was added, were assayed after four hours at 4° C.

RESULTS

Sterilization and Potency.—

1. *Dry antibiotics.*—a. *Penicillin containing B. globigii spores:* Samples of penicillin exposed at room temperature to ethylene oxide vapor (475 mg. per liter) for six hours were completely sterile. Similar samples not treated with ethylene oxide were found to contain approximately 1,500,000 viable spores.

Potency of the penicillin in those containers exposed to ethylene oxide was determined, and compared with unexposed samples to which spores had also been added. Results obtained in one of many such tests are given in Fig. 1; similar results were observed throughout. The difference between exposed and unexposed samples appears to be less than the deviation of the mean of either, at all levels assayed. (The diameter of the circle represents the average deviation.) We conclude that the potency of penicillin as measured this way is not affected by the sterilization procedure. Samples of the same lot, which were not contaminated with microorganisms, gave the same potency results; apparently the presence of dead organisms does not affect the bacteriostatic activity of these preparations.

b. *Penicillin containing E. coli:* Samples containing one thousand units of dry penicillin were exposed at room temperatures to ethylene oxide vapor (475

*Schenley Laboratories, Inc., Lawrenceburg, Ind.

mg. per liter) for two and for four hours. Complete sterilization was affected in two hours, while untreated samples had an average concentration of approximately 38,000,000 bacteria.

Assay of the penicillin indicated no significant decrease in potency.

c. *Penicillin containing A. tamarii spores:* Dry penicillin containing spores of *Aspergillus tamarii* was exposed for two and for four hours under the same conditions as above. A great reduction was observed after two hours of expo-

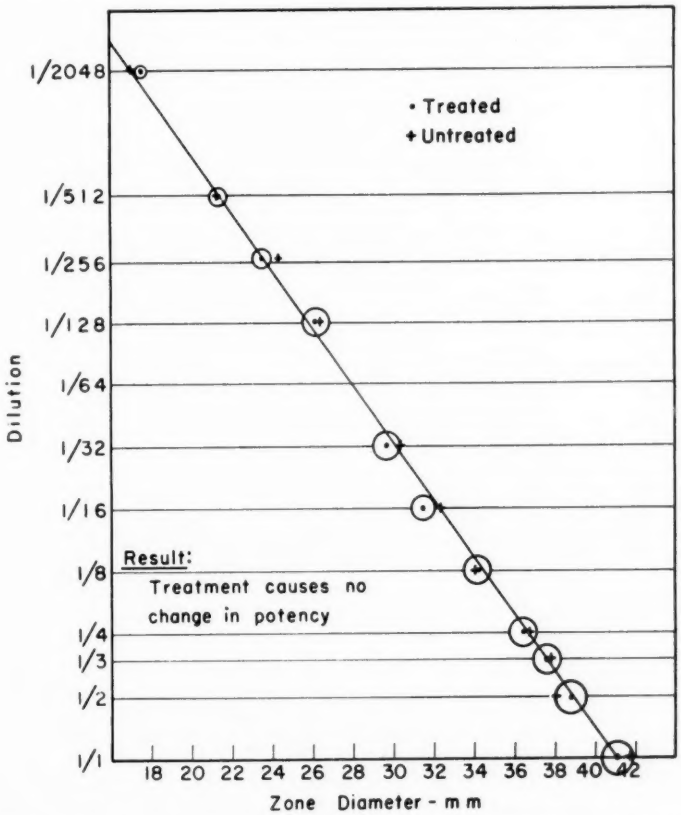

Fig. 1.—Assay of penicillin sterilized by exposure to ethylene oxide (475 mg. per liter for six hours at 25° C.).

TABLE I. SURVIVAL AT 4° C. OF E. COLI AND B. GLOBIGII SPORES IN PENICILLIN SOLUTIONS CONTAINING ETHYLENE OXIDE

ETHYLENE OXIDE CONC. (VOL. %)	TIME OF EXPOSURE AT 4° C. (HOURS)	NUMBER OF ORGANISMS SURVIVING PER ML. SOLUTION	
		E. COLI	B. GLOBIGII
0	4	2.4×10^9	5.7×10^7
4.8	2	8.2×10^6	2.2×10^7
	4	1.4×10^6	4.0×10^6
9.1	2	40	8.2×10^5
	4	0	8.4×10^4
16.7	2	0	4.3×10^2
	4	0	0

sure, while four hours gave complete sterilization. The exposed material was unchanged in potency. Untreated penicillin-fungus mixtures contained approximately 2,000,000 spores.

d. *Streptomycin:* The results of some typical potency tests presented in Fig. 2 indicate that treatment with ethylene oxide causes a 35 per cent reduction in the potency of streptomycin. When this inactivation was confirmed, no sterilization trials were attempted.

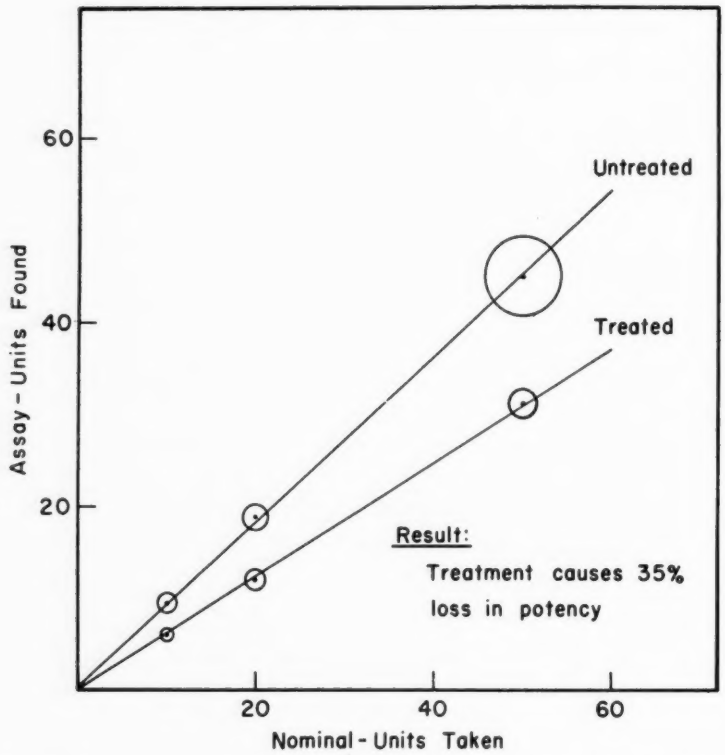

Fig. 2.—Assay of streptomycin sterilized by exposure to ethylene oxide (475 mg. per liter for six hours at 25° C.).

2. *Penicillin solutions.*—Results of these tests are sumarrized in Table I.

It is apparent that even at refrigerator temperature solutions containing great numbers of highly resistant spores may be rendered sterile.

The potency of penicillin solutions treated in this manner was determined and was found to be identical with that of untreated material. It is, furthermore, easy to remove ethylene oxide from such solutions after sterilization is complete, either by simple warming of cotton-plugged containers[7, 10] or by aeration.

Acute Toxicity of Treated Antibiotics.—

1. *Penicillin.*—All mice receiving 30,000 units (1,500,000 units per kilogram) of untreated and treated penicillin at first showed signs of sluggishness,

interrupted intermittently by greatly increased activity. Recovery from this effect was rapid; no deaths occurred in either group. It appears that no great increase in acute toxicity was imparted to the penicillin by the six-hour ethylene oxide treatment.

2. *Streptomycin.*—All mice receiving the 10,000 units of streptomycin, both treated (six hours) and untreated, at first showed signs of sluggishness and respiratory difficulty. One mouse died in each group; postmortems indicated mottled kidneys and white-tinted livers. All other mice in both groups recovered rapidly. Ethylene oxide treatment thus appeared to cause no increase of acute toxicity of streptomycin.

DISCUSSION

The moisture content of the dry samples treated was not determined. It has been pointed out[4] that extreme dryness leads to occasional "skips" in the sterilization of materials by ethylene oxide. Apparently, the lyophilization procedure did not yield materials containing less than the critical moisture content.

The fact that penicillin was unchanged in antibiotic potency, but that streptomycin was definitely deteriorated by ethylene oxide treatment, suggests that tests of this nature must be conducted with each antibiotic to which the procedure is applied.

The method of application of ethylene oxide may, of course, be varied to suit individual needs.[1] Prior brief evacuation aids penetration, but may result in too dry a product. Ethylene oxide and mixtures containing this material must be handled with care,[1, 6] but present no real problem when their nature is understood.

SUMMARY

Penicillin may be sterilized by exposure of the dry powder to ethylene oxide vapor or by adding this chemical to solutions of the antibiotic. No loss in potency or increase in acute toxicity results from such treatments.

Streptomycin, calcium chloride salt, however, appeared to lose 35 per cent of its potency when treated with ethylene oxide vapor. No increase in acute toxicity was noted.

Grateful acknowledgment is made of the valuable assistance rendered by the following personnel of Camp Detrick: Dr. Riley A. Housewright, Dr. Joseph Forgacs, Mr. Bruce H. Morgan, and Mr. Edward Cherry.

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CLASSIFICATION AND DISTRIBUTION OF 1,075 CULTURES OF SALMONELLA ISOLATED IN THE CITY OF MEXICO

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ONE THOUSAND SEVENTY-FIVE cultures of Salmonella, isolated from man and from domestic animals, have been studied at the Salmonella Center of Mexico. The human and animal samples other than blood and feces were inoculated in enrichment Kauffmann's medium. The fecal material was plated in S S Agar (Difco) in addition to the enrichment medium. This medium was incubated for twenty to forty-eight hours and plated in S S Agar (Difco) and brilliant green agar. Negative lactose colonies were summited to a small group of biochemical reactions (glucose, lactose, sucrose, mannitol, urea, H_2S, and indol).[6] The blood was cultured following the usual techniques. Those cultures with biochemical characteristics of the genus *Salmonella* were grouped with "O" and typed with "H" *Salmonella* sera according to the methods described by Kauffmann,[5] and by Edwards and Bruner.[1]

Table I presents a summary of the distribution by groups, in accordance with the serological classification of Kauffmann-White,[5] and by the origin of the cultures. Fifty-eight species or types of Salmonella were found.

Of these fifty-eight serological types, sixteen represent 85 per cent of the total strains studied. With the exception of *S. typhi*, *S. paratyphi A.*, *S. paratyphi B.*, and *S. enteritidis*, these sixteen types showed the highest incidence both in man and in animals (Table I). *S. typhi* was isolated 244 times from man and two times from milk samples; *S. paratyphi A.* Twenty-one times from man and one time from a milk sample; *S. paratyphi B.* was found ten times in man, one time in a pig, and five times in milk samples; *S. enteritidis* was isolated seven times from man and forty times in animals.

Table II comprises those types whose incidence was higher than 30 per 1,000, corresponding to the following Salmonellas: *derby, typhi-murium, cholerae-suis, muenchen, newport, typhi, enteritidis, anatum*, and *newington*.

Table III comprises those Salmonellas with an incidence higher than 10 and lower than 30 per 1,000: *paratyphi A., paratyphi B., chester, montevideo, oranienburg, new-brunswick*, and *poona*.

Of the 706 cultures of human origin, 487 corresponding to forty-four different Salmonellas were isolated from stool samples; 169 were found by blood culture, with only four types; *S. typhi* (155 times), *S. paratyphi A.* (Seven times), *S. typhi-murium* (five times), and *S. paratyphi B.* (two times). Fifty Salmonellas classified in fourteen types were isolated from different human products other than blood or feces.

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TABLE I

SALMONELLA	MAN								ANIMALS																
									PIGS					COWS			RATS							CERTIFIED MILK 13	TOTALS
	ADULTS (STOOLS) 6, 8, 12	CHILDREN STOOL 6, 8	MESENTERIC LYMPH NODES 9	TONSILS 12	BLOOD	CEREBROSPINAL FLUID	OTHER TISSUES	TOTAL	MESENTERIC LYMPH NODES	LIVER	INTESTINE	SPLEEN 12, 13	OTHER TISSUE	SPINAL CORD	SPLEEN	LIVER 13	SPLEEN	INTESTINE 10	MICE (SPLEEN) 12	CHICKENS 14	DOGS 11	TOTAL		
Paratyphi A.	10	4			7			21														1	1	22
Paratyphi B.	3	13			2			18					1									1	5	24
Stanley	1							1																1
Chester	1	5	1	1				8					2							2	1	5	3	16
Derby	8	28		3				39	1	35	3	4	13	1	5	3				1	5	71	6	116
Essen	1		1					2																2
Typhimurium	22	67	5	2	5	2	2	105	2	1			4				1	3	75	5		91	2	198
Bredeney	1							1																1
Reading	1	1	1					3																3
Abony	1							1							1								1	2
Schleissheim											1											1		1
Heidelberg									1													1		1
Texas	1							1																1
Paratyphi C.		3						3																3
Cholerae-suis	4	6	1					11	3				15				2		1	2		23		34
Montevideo	1	6	3					10												1	2	3		13
Thompson	3	6						9		1												1		10
Daytone	1	1						2																2
Oranienburg	3		6					9		1				1								2		11
Muenchen (var. Oregon and var. Mexicana)	4	14	4	3				25		2	1				1					1		5	1	31
Manhattan	2							2																2
Newport	14	30	4	1				49			1	1	1		7		1			1		12	2	63
Litchfield		1						1																1
Bovis-morbificans		1						1							1							1	1	3
Braenderup	1	1						2																2
Glostrup		1						1																1
Bonariensis	1							1																1

Typhi	47	40			155	1	1	244															2	246
Eatsbourne	2	2					1	5																5
Berta	3							3																3
Enteritidis	3	4						7									11	4				15		22
Enteritidis var. daniz (Pensacola var. anahuac)																	25					25		25
Pensacola																	9	1				10		10
Panama	2			3				5							1	2						3		8
Pullorum	2							2																2
Sendai	2							2																2
Oranimon									1													1		1
Rostock							1	1													1	1		2
Vejle																1						1		1
Anatum	31	20						51	6			1	9	1	2			1		1		21		72
Meleagridis									1												1	2		2
London	3							3					1								2	3		6
Give	1	1						2							2						2	4		6
Newington	3	17						20		5	2	1	2	3	1	2				5	2	23		43
Selandia										1			1	2	3							7		7
New-Brunswick	3	4						7					1		2						2	5		12
Senftenberg	1	2						3	1													1	1	5
Illinois																					1	1		1
Aberdeen							1	1																1
Poona	1	11						12	1													1		13
Onderstepoort	1							1																1
Carrau		1	1	2				4																4
Gaminara		1						1																1
Minnesota	1							1																1
Urbana	1	2						3												2		2		5
Rubislaw	1	1						2					1									1		3
Cerro																					1	1		1

TABLE II. SALMONELLAS WHICH PRESENTED AN INCIDENCE HIGHER THAN 30 PER 1,000

1	S. derby	116
2	S. typhi-murium	198
3	S. cholerae-suis	34
4	S. muenchen	31
5	S. newport	63
6	S. typhi	246
7	S. enteritidis	47
8	S. anatum	72
9	S. newington	43

TABLE III. SALMONELLAS WHICH PRESENTED AN INCIDENCE HIGHER THAN 10, AND LOWER THAN 30 PER 1,000

1	S. paratyphi A	22
2	S. paratyphi B	24
3	S. chester	16
4	S. montevideo	13
5	S. oranienburg	11
6	S. new-brunswick	12
7	S. poona	13

The presence of Salmonella was investigated in seven groups of domestic animals, 368 cultures were isolated, corresponding to thirty-three types distributed as shown in Table I. As may be seen in Table IV, a large number of animals was found infected with these bacteria.

TABLE IV. INCIDENCE OF SALMONELLA PER ONE THOUSAND ANIMALS

Pigs in apparent normal condition[12]	70
Pigs with enteritis[12]	733
Cows in apparent normal condition[13]	106
Rats in apparent normal condition[10]	105
Mice, spontaneously dead[12]	250
Chickens in apparent normal condition[14]	13
Dogs (clinical condition unknown)[11]	90

Twenty-four Salmonellas of ten different types were isolated from 520 samples of certified milk.[13]

Although up to the present time about 200 species or types of Salmonella have been described, this small group of sixteen (Tables II and III) is the only one which has epidemiological interest. Its distribution and prevalence is similar in the whole American Continent, as shown by this communication and by publications of Edwards, Bruner, and Moran,[2] and Seligmann, Saphra, and Wassermann,[7] in the United States of America; Hormaeche, Surraco, Peluffo, and Aleppo[4] in Uruguay; and Henderson[3] in Panama.

In the classification of cultures, we used type sera received from Dr. F. Kauffmann, Director of the International Salmonella Center, Copenhagen, Denmark. Some of the Salmonella were identified or confirmed by Dr. E. Hormaeche of Montevideo, Uruguay, and by Dr. P. R. Edwards, Communicable Disease Center, Atlanta, Georgia, U. S. A. The authors express their gratitude to all these persons.

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A STUDY OF ORGANISM *t* ANTIGEN IN THE ROUTINE COMPLEMENT FIXATION TEST FOR AMEBIASIS

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FROM time to time the question of the specificity of the complement fixation test for amebiasis has arisen because of the lack of agreement between positive serological results and clinical findings. Craig[1] recognized this fact and stated, "Unfortunately the complement fixation test for amebiasis is not perfected at present and, as with most complement fixation tests, false positive and false negative tests may be obtained." The antigen used by Craig for the amebiasis complement fixation test was an alcoholic extract of the ameba grown with a heterogeneous mixture of bacteria. Craig stated, "It has been repeatedly demonstrated that such contamination does not cause false positives."

Rees, and associates[2] produced antigens from cultures of *Endamoeba Histolytica* grown in the presence of a single bacterial symbiont called organism *t*. The specificity of the antigen was studied by preparing separate rabbit antisera against organism *t* and against *E. histolytica*. They carried out complement fixation tests using these antigens against their homologous antisera, and obtained positive reactions of high titer. With the heterologous system, however, their data showed that a 1:2 dilution of organism *t* antigen reacted with a 1:2 dilution of *E. histolytica* antiserum. Since their antigen preparations were sufficiently active to be used in actual testing at the 1:8 dilution, the possibility of nonspecificity with less potent antigens for the routine diagnosis of amebiasis was not discussed. This question is of some concern to us, because at times it has been necessary in our laboratory to use some ameba antigen preparations in dilutions as low as 1:4.

It is the purpose of this paper to describe results from the parallel use of a control antigen prepared with a pure culture of organism *t* and the antigen prepared from *E. histolytica* plus organism *t* in the routine serological test for amebiasis, and to investigate the desirability of including an antigen *t* control in routine testing.

METHOD

Serum specimens routinely submitted for the complement fixation test for amebiasis were tested with antigen** made from amebas and organism *t*, grown on Boeck and Drbohlav's medium and prepared in general according to the method of Rees.[3] Three antigen prepara-

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**The antigens used in this study were prepared by Miss Lois Norman, Laboratory Branch, C.D.C.

tion were used in this study: (1) antigen prepared from *E. histolytica* strain 103 cultured with organism *t*, referred to as *ameba antigen;* (2) antigen prepared from organism *t* alone, referred to as *t* antigen; and (3) antigen prepared from the uninoculated medium, referred to as *medium antigen.*

The titer of the ameba antigen as used in this study was 1:6. Accordingly, the *t* antigen control was used at the same dilution. Additionally, antigen *t* was used at 1:4 dilution in order to determine if the reaction with *t* would be greater than that obtained with 1:6 dilution. Medium antigen was used at a dilution of 1:4.

The complement fixation method, as developed by Bozicevich and associates,[4] was somewhat modified for this study. In this test, sera were diluted 1:5 and were tested with four 50 per cent hemolysis units of complement.

The dilution of antigen for the test was determined from checkerboard titration with a serum which was positive for amebiasis by clinical and laboratory findings. Tests were made with two units of antigen. A barbiturate saline buffer was used instead of the phosphate buffer of the original method. The hemolytic system was added after the tests had been incubated for fifteen to eighteen hours at 4 to 6° C. The degree of hemolysis was read with the aid of prepared hemolysis standards. The results were recorded as negative, plus-minus, 1 plus, 2 plus, 3 plus, 4 plus. A 2 to 4-plus reading was considered a strong reaction with *t* antigen, whereas 3 or 4 plus was reported as positive with ameba antigen.

Absorption experiments were carried out with bacteria from a pure culture of organism *t* grown for forty-eight hours in thioglycolate broth. A large yield of organism *t* was obtained and was removed by centrifugation. This was resuspended in saline and again centrifuged. Absorptions were made with 0.5 ml. of serum and 0.5 ml. of *t* organisms incubated in the water bath for two hours at 37° C. Human sera which had shown positive reactions with ameba antigen and with *t* antigen were absorbed.

RESULTS

The results of the complement fixation tests on 490 specimens submitted for routine examination are given in Table I. In this table, the degree of complement fixation with a 1:5 dilution of serum is recorded in terms of minus or plus marks. It can be seen that, of the 66 specimens positive 3 plus with ameba antigen, 10 reacted 2 plus or better with antigen *t* used 1:4, and 3 reacted 2 plus or better with antigen *t* used 1:6. Of the 68 specimens positive 4 plus with ameba antigen, 17 reacted 2 plus or better with antigen *t* used 1:4 and 5 reacted 2 plus or better with antigen *t* used 1:6. One specimen was positive (4 plus) with the medium antigen.

An additional group of 886 sera, not shown in the table, were tested for amebiasis with a new lot of antigen, whose titer was 1:8. This was controlled

TABLE I: AMEBA ANTIGEN REACTIONS OBTAINED WITH 490 SERA SUBMITTED FOR THE AMEBIASIS COMPLEMENT FIXATION TEST, IN COMPARISON WITH T AND MEDIUM ANTIGENS

AMEBA ANTIGEN (USED 1:6)		REACTION OF SERA WITH T ANTIGENS (USED 1:4)						REACTION OF SERA WITH T ANTIGENS (USED 1:6)						REACTION OF SERA WITH MEDIUM ANTIGEN (USED 1:4)					
NO. SERA	REACTION	NO. NEG.	±	1+	2+	3+	4+	NO. NEG.	±	1+	2+	3+	4+	NO. NEG.	±	1+	2+	3+	4+
128	Neg.	123	5					128						128					
102	+	79	21	1	1			91	11					98	4				
63	1+	37	22	4				57	6					59	4				
63	2+	43	14	3	3			50	7	6				60	3				
66	3+	37	14	5	1	9		58	3	2	3			63	3				
68	4+	34	8	9	7	6	4	47	11	5	3	1	1	66	1				1

with the *t* antigen diluted 1:8. In this group, 183 were positive with the ameba antigen. The results with *t* antigen showed that a 1 plus reaction was obtained with 14 specimens, a 2 plus reaction with 2 specimens, and a 4 plus reaction with 2 other specimens. No reaction was obtained with the medium antigen.

A study was made to determine the effect of dilution of the serum upon the reaction of *t* antigen and ameba antigen with 23 specimens positive at the 1:5 dilution with both antigens. Dilutions of 1:5, 1:10, and 1:20 were made in barbiturate buffered saline. At the 1:10 dilution, 10 were positive with the ameba antigen alone; 3 were positive with the *t* antigen alone; and 10 were negative with both antigens. None of these specimens were positive at the 1:20 dilution.

A study of 8,724 other specimens negative with the ameba antigen showed that only three of these specimens were positive with antigen *t* alone.

It was not possible to demonstrate distinct serum components for organism *t*, even when multiple absorptions were made. Human sera absorbed with organism *t* still reacted with both the ameba antigen and with *t* antigen, but in slightly lower titer.

DISCUSSION

It was found that of 134 specimens positive (3 or 4 plus) with the ameba antigen, 24 specimens reacted 2 plus or better with *t* antigen used 1:4, and 8 specimens reacted 2 plus or better with *t* antigen used 1:6. Although a 2 plus reaction was not reported as positive for amebiasis, it was considered significant when testing with antigen *t*.

In the preparation of both the ameba and organism *t* antigens, some of the nonspecific antigenic constituents of the basal medium may persist. It is suspected, since only 3 specimens of 8,724 were positive with *t* antigen alone, that some nonspecific reactions might be due to the medium components. The use of a control of *t* antigen will also control nonspecificity due to the medium.

It was demonstrated that reactions with organism *t* have been eliminated or reduced by diluting the sera. In some cases, however, it was not possible to eliminate the reaction with antigen *t* by diluting before the reaction with ameba antigen disappeared. This indicated either that a false positive result was obtained with the ameba antigen or that the titer due to *E. histolytica* infection was too low to be differentiated from the *t* antigen by dilution.

Since it was not possible to absorb completely that part of the sera which reacted with the *t* antigen, it could be postulated that these reactions with *t* antigen were of a nonspecific nature caused by an antigen common to *E. histolytica* and organism *t*.

CONCLUSIONS

The data presented in this study showed that possibly nonspecific results were obtained with the antigen prepared from ameba grown in the presence of organism *t*. Accordingly, an organism *t* control should be included in the complement fixation test for amebiasis to help eliminate or identify such false positive reactions with ameba antigen prepared and used as described. Since antigens used at 1:8 dilution gave little reaction wth organism *t*, as compared to the

1:4 dilution, it may be assumed that ameba antigens of still greater potency could be used wthout controls for organism *t*. Ameba antigens which titered to 1:64 were recently obtained from a laboratory supply house. However, organism *t* was not available for parallel testing with the commercial antigen to clarify this point.

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THE OCCURRENCE OF BACTEREMIA AND DEATH IN CORTISONE-TREATED MICE

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DURING a prior experiment designed to determine the effect of cortisone on antibody production following intraperitoneal injection of PR8 influenza vaccine, it was observed that 57 of 88 cortisone-treated mice died between the fifth and sixteenth days of treatment. At autopsy, nodular lesions were seen in the viscera of 47 of the 57 dead mice. No deaths occurred in a group of 88 saline-treated mice. The present paper represents the results of studies on the incidence of deaths and bacteremia in mice during the course of prolonged cortisone treatment.

METHODS

In each experiment, test and control mice of similar ages and weights (18 to 23 Gm.) were housed together. Test mice received daily subcutaneous injections of cortisone acetate.* Control mice received subcutaneous injection of physiological saline solution daily. After various intervals of treatment, the animals were sacrificed to obtain cultures, after which autopsies were performed. Cultures of heart's blood and liver or spleen were obtained only from animals which had been sacrificed. Aerobic culture methods were utilized. Mice were selected for killing, with no attempt being made to choose ill- or well-appearing animals.

Tissue sections were obtained only from sacrificed animals, and were fixed in Zenker's, Bouin's, or alcohol-formalin fixative. Tissues were stained with hematoxylin-eosin, Best's carmine, sudan III, Hotchkiss-McManus, or Weigert's gram stain.

It should be emphasized that no controlled laboratory infection was induced in the mice. To verify that no infection was induced in the test mice, 0.05 ml. of material from each of several of the bottles of cortisone used in the experiments was cultured in 20 ml. of thioglycollate media. No evidence of aerobic or anaerobic growth was noted in these cultures.

EXPERIMENTAL RESULTS

EFFECTS OF CORTISONE

The usual indications of cortisone activity were noted in the test mice. These were (a) a depression of the total white blood cell count and elevation of the per cent of polymorphonuclear cells, (b) a marked and progressive decrease in weight, and (c) a decrease in spleen size to one-half or less the size of spleens in control mice.

The following observations were considered to be additional evidence of cortisone activity:

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Liver.—At autopsy, the livers of test mice grossly showed a wide variation in the amount of yellow change. Microscopically, there were marked changes in the liver parenchymal cells. There was a variation in the size of nuclei. The cytoplasm of parenchymal cells was swollen and contained large amounts of glycogen deposited surrounding the nucleus. These changes appeared diffusely through the liver lobule. No abnormalities of the biliary passages or reticuloendothelial system were noted.

Adrenals.—Except for a mild vacuolization of the cells in the zona glomerulosa and zona fasciculata in the cortices of test mice, no differences were found between the adrenal glands of test or control mice.

Intestinal tract.—On gross examination, no significant difference was seen between the intestinal tracts of test or control mice. In one experiment in which ten test and ten control mice were treated for ten days and then sacrificed, particular attention was paid to the examination of the intestinal tract. In that group, one test mouse was found to have a reddened serosa and engorgement of the blood vessels of the distal portion of the small intestine.

In addition, deaths, bacteremia, and nodules appeared during the course of cortisone treatment as follows:

Deaths.—As seen in Table I, a total of 41 spontaneous deaths occurred among 167 test mice. Only one death occurred among 165 control mice. A true death rate in either of these groups cannot be given, since animals were sacrificed at various intervals after beginning cortisone treatment. However, of those animals not sacrificed, 50 per cent of the mice receiving 1.25 mg. of cortisone had died by the twelfth day of treatment, and 50 per cent of the mice receiving 0.75 mg. of cortisone had died by the seventeenth day of treatment.

Bacteremia.—To avoid postmortem contaminants, none of the mice dying spontaneously were cultured for evidence of bacteremia. As seen in Table I,

TABLE I. SUMMARY OF FINDINGS IN CORTISONE-TREATED MICE AND SALINE-TREATED MICE

	NO. OF MICE	DEATHS NO. OF SPONTANEOUS	NO. OF MICE POSITIVE CULTURES/ CULTURED	NO. OF MICE WITH NODULES	DURATION OF CORTISONE TREATMENT (DAYS)
		Group A—Rockland Farm ♀ 0.75 mg. cortisone daily			
Test	68	21	17/47*	38	9-28
Control	67	0	6/64	0	
		Group B† ♀ *0.75 mg. cortisone daily*			
Test	13	3	5/10	7	13-26
Control	12	0	1/11	0	
		Group C—Carworth Farms ♂ 1.25 mg. cortisone daily			
Test	33	6	4/27	6	0-8
Control	33	0	0/27	0	
		Group D—Carworth Farms ♂ 1.25 mg. cortisone daily			
Test	53	11	30/40‡	7	7-14
Control	53	1	1/40	1	
Totals:					
Test	167	41	56/124	58	
Control	165	1	8/142	1	

*In three of these mice only positive spleen culture was obtained.
†Raised at this hospital.
‡In eight of these mice only positive liver cultures obtained.

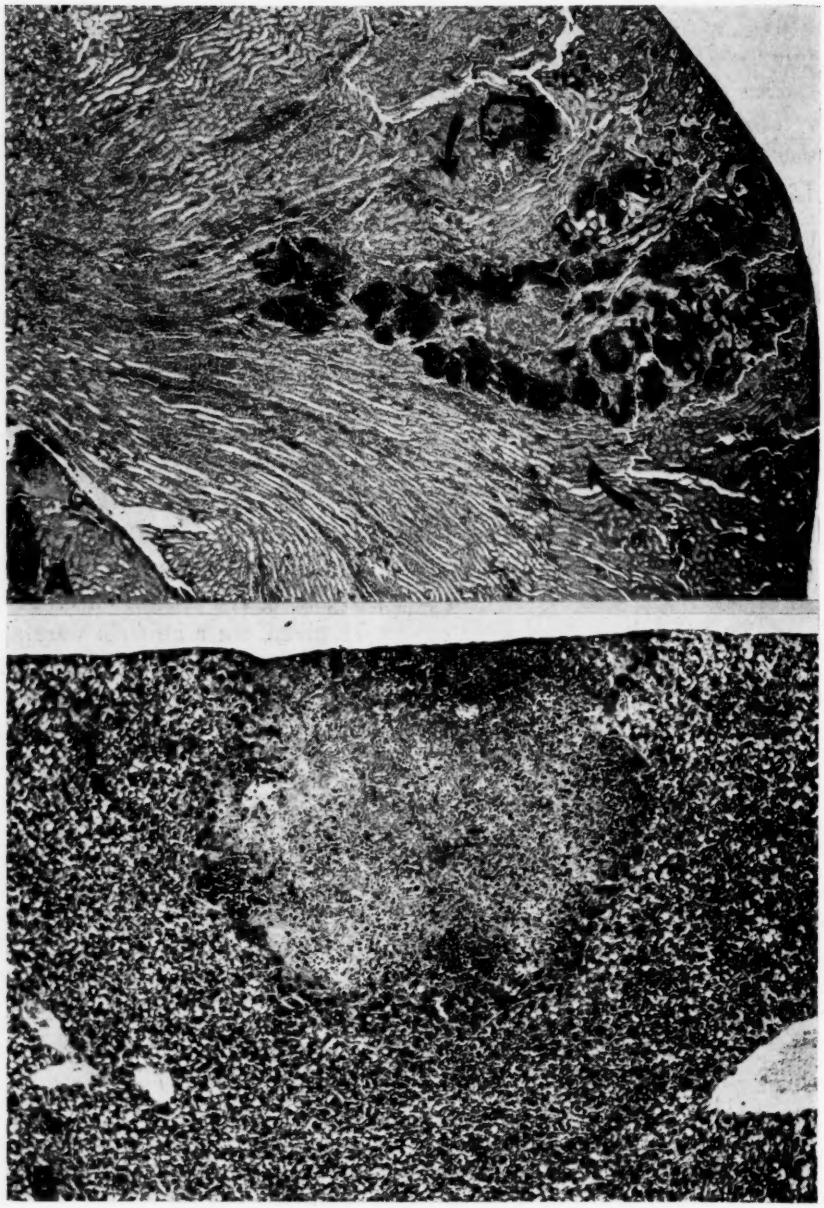

Fig. 1.—*A*, Low-power (×70) magnification of a lesion in the cortex of the kidney suggestive of an embolic phenomenon. (Hematoxylineosin stain of Zenker-formalin fixed tissue). *B*, Low-power (×25) magnification of a focal necrotic lesion in the liver. Central necrosis, mild inflammatory reaction, and growth of microorganisms, in that order, are seen as fairly distinct concentric zones. (Hematoxylin-eosin stain of Zenker-formalin fixed tissue).

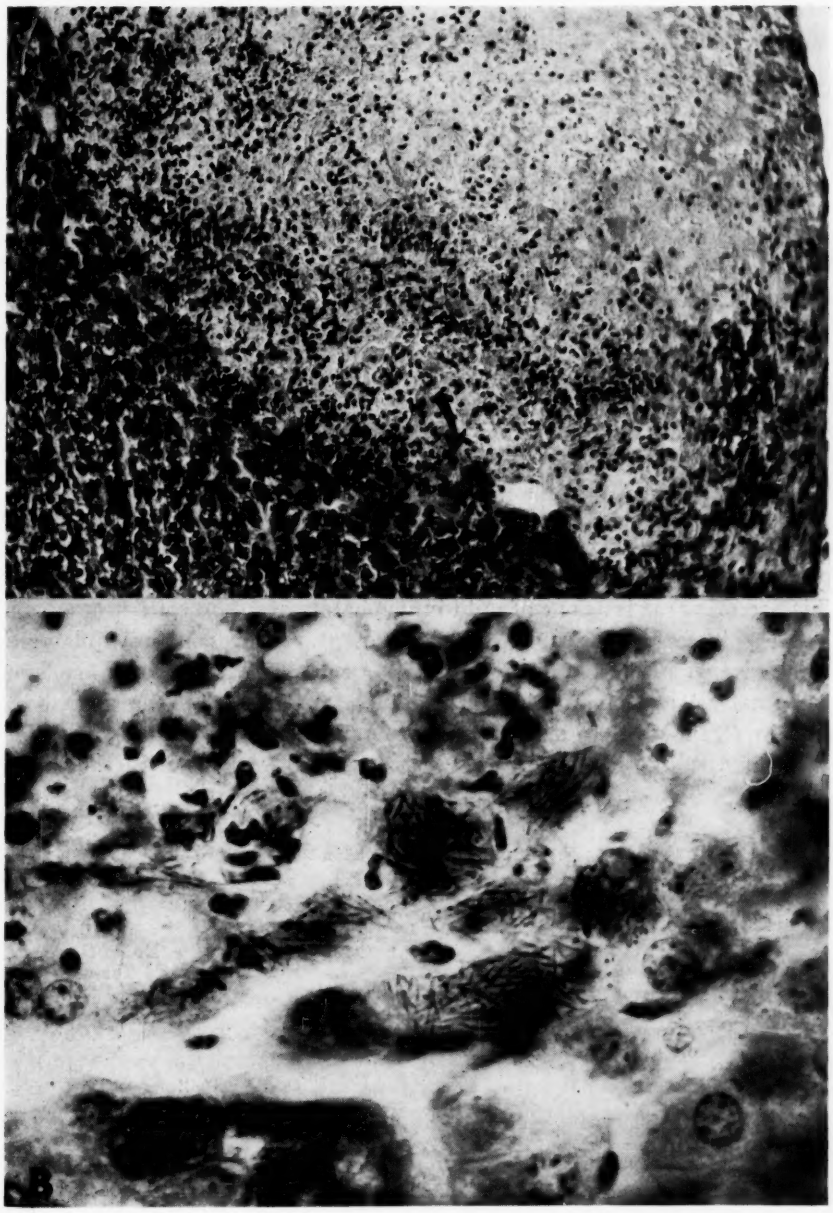

Fig. 2.—*A*, Moderate (×115) magnification of the focal necrotic lesion in the liver. The three zones described in the text are seen in greater detail. (Hematoxylin-eosin stain of Zenker-formalin fixed tissue). *B*, High-power (×1530) magnification of most peripheral zone of the focal necrotic lesion in the liver showing the detail of this deeply staining zone. Microorganisms of varying shapes are seen intra- and extracellularly. Hotchkiss-McManus hematoxylin stain of Zenker-formalin fixed tissue).

56 of 124 test mice as compared to 8 of 132 control mice had evidence of a bacteremia as determined by a positive heart's blood, liver, or spleen culture. Up to the fifth day of treatment, at which time one of the three test animals autopsied showed evidence of a bacteremia, no bacteremia was noted in 15 test or 15 control mice. Thereafter, the incidence of bacteremia in the test group of mice increased with more prolonged treatment.

The bacteremia consisted of gram-negative bacteria. Organisms which were recovered from 27 of 37 test mice and one of 36 control mice, included in the preceding figures, are further characterized in Table II.

Nodules.—All mice, including those which died spontaneously and those which were sacrificed, were autopsied and examined grossly. As seen in Table I, 58 of 167 test mice showed grayish nodular lesions in one or more of the viscera. One of 165 control mice was found to have a solitary nodule at the time of sacrifice. These grayish nodular lesions, approximately 1 to 2 mm. in diameter, were raised and sharply defined from the tissues of the liver and kidney in which they most frequently occurred. On cut section the lesions appeared homogenous and did not show liquefaction (Fig. 1, *A* and *B*).

Microscopically (Fig. 2, *A*), the nodule in the liver appears as a large focal necrotic area, centrally showing cellular and nuclear debris. A few bacteria and strands of fibrin can be seen on the Gram-Weigert stained section. Surrounding this necrotic area is a zone of mild inflammatory reaction consisting of plasma cells and polymorphonuclear cells. Peripheral to the zone of inflammatory reaction, groups of long, narrow, beaded, or clubbed organisms are seen in parenchymal cells and sinusoids (Fig. 2, *B*). These organisms appear gram-positive in Gram-Weigert stained section, and are stained red by the Hotchkiss-McManus stain. There is a sparse inflammatory reaction in the region of the organisms.

TABLE II. BACTERIOLOGIC FINDINGS IN CORTISONE-TREATED AND SALINE-TREATED MICE

Number of cortisone-treated mice sacrificed			37
Number of these mice showing bacteremia			27*
Organism:		No. of times found	
Coli-aerogenes group		12	
Other gram-negative rods which grow on E.M.B.:			
Proteus	2		
Pseudomonas	2		
Lactose—negative, H_2S—negative	15		
Lactose—negative, H_2S—positive	1		
Total		20	
Number of saline-treated mice sacrificed			36
Number of these mice showing bacteremia			1
Organism:			
Streptococcus fecalis		1	

*In five treated mice, more than one organism was found.

Cultures of nodules which were found in three of eight test mice having nodular lesions yielded Corynebacterium organisms.

DISCUSSION

Since the introduction of ACTH and cortisone as therapeutic agents, many authors (as reviewed by Kass and Finland[1]) have reported diminished resistance to naturally occurring and experimental infections in both human beings and animals treated with these drugs. Moreover, Antopol and associates,[2, 3] reported spontaneous deaths and the occurrence of "granulomatous" nodules in the viscera of "normal" mice which had been given large amounts of cortisone. From these nodules he cultured Corynebacterium organisms. Ingle and associates[4, 5] noted deaths and the occurrence of nodules in "normal" rats after treatment with cortisone or ACTH, but he did not report bacteriologic observations. Thomas and Good[6] have reported the occurrence of bilateral cortical necroses of the kidneys of rabbits which had been inoculated with single doses of meningococcal or *S. marcesens* toxins after receiving large doses of cortisone.

The data presented in this paper not only confirm Antopol's observations, but also describe a bacteremia in cortisone-treated mice. This finding of a bacteremia consisting of organisms which are regarded as normal inhabitants of the gastrointestinal tract of the mouse[7] emphasizes the significance of the alteration of the host-parasite relationship which occurs in "normal" laboratory animals treated with large doses of cortisone. The finding of intestinal bacteria also adds weight to Antopol's suggestion[3] of a parallelism between the effects of cortisone and the effects of total body x-radiation on the animals' resistance to infection.[8, 9]

The lowering of host resistance to infection may be due to the effects of cortisone on natural protective mechanisms. For example, cortisone has been shown to decrease the inflammatory reaction against intraperitoneally injected India ink,[10] or oil,[11] and cutaneous irritation by nonspecific irritants.[12] The mild reaction apparent in the liver section (Fig. 2) is illustrative of the depression of the inflammatory reaction. Furthermore, a depression of the phagocytic activity of neutrophilic leukocytes obtained from human beings treated with cortisone has been reported.[13] This depression of phagocytosis might be explained by Vaughn's[14] observation that a fall of human complement titers results from the administration of ACTH.

The natural mucosal barrier of the stomach has been observed to undergo ulceration in animals[4, 5] and human beings[15-17] which were treated with ACTH or cortisone. In this study, no such disturbances of this protective mechanism were noted on superficial examination.

On the other hand, the lowering of host resistance to infection may be due to the effect of cortisone on acquired protective mechanisms. However, in work published up to this time, neither ACTH nor cortisone seems to depress the level of acquired immunity to infectious agents.[18-23]

Increased susceptibility to infection has been stressed as one of the clinical complications of prolonged cortisone therapy.[1, 24, 25] The occurrence of a bac-

teremia might possibly explain the presence of a disseminated infection in patients who have been treated with cortisone.[26]

It is interesting to note that Selye[27, 28] has reported that the growth hormone (STH) antagonizes the depression of resistance to infection induced by cortisone.

SUMMARY

Cortisone was administered subcutaneously to "normal" laboratory mice with the following results: (1) Animals treated with large doses of cortisone died and at autopsy showed focal necrotic areas in the viscera. (2) A bacteremia was found in cortisone-treated animals. This bacteremia consisted of organisms generally regarded as normal inhabitants of the gastrointestinal tract.

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A STUDY OF THE COMPLEMENT FIXATION REACTION IN HISTOPLASMOSIS*

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THE concept of histoplasmosis as an almost uniformly fatal disseminated fungus infection[1] has been dispelled by the appearance of an increasing number of proved cases which have recovered.[2-16] In addition, the presence of a benign form of pulmonary infection with *Histoplasma* is being recognized as the result of extensive epidemiological investigation.[17-20] Establishing the diagnosis in cases of severe generalized histoplasmosis has been practical when the fungus etiology is suspected. Culture of blood, bone marrow, sputum, and biopsy tissue will frequently yield *Histoplasma* organisms. Failing in this, biopsy or post-mortem tissue sections usually show a characteristic picture. Proving the diagnosis in milder infections has been difficult.[21-24] The few cases of mild or asymptomatic *Histoplasma* infections from which the organism has been recovered represent in most cases findings from extensive search.[13, 24] The differential diagnosis of pulmonary infiltrates, especially in the midwestern United States, must include histoplasmosis. The usefulness of the histoplasmin skin test in this connection is limited by the high percentage of reactors in the normal population of this region. The difficulty of isolating the organism from sputum in pulmonary histoplasmosis is well known.[22] Even in cases of severe generalized infection isolation of *Histoplasma* takes considerable time and requires experienced personnel. It is apparent that any aid in the diagnosis of histoplasmosis would be most helpful.

In an effort to fill this need serological tests have been introduced which attempt to discover active cases of histoplasmosis. Such tests have proved of value in other systemic mycoses. Smith and associates,[25] in a wide experience with coccidioidomycosis, have been able to draw important diagnostic and prognostic conclusions from precipitin and complement fixation tests employing coccidioidin as antigen. The complement fixation test has likewise proved of diagnostic and prognostic significance in blastomycosis.[26] Serological tests have been used less extensively but to advantage in other mycotic infections.[27, 28]

Several different serological tests for histoplasmosis have been introduced. Salvin[29] reported a complement fixation test with whole formalin-killed yeast cells as antigen that

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demonstrated antibodies in sera from experimentally infected rabbits appearing within two weeks after infection and persisting for twenty-three weeks. Salvin and Hottle[30] then investigated the suitability of various yeast-phase and mycelial-phase *H. capsulatum* preparations as antigen in complement fixation and precipitin tests. They used sera from experimentally infected and hyperimmune rabbits. Histoplasmin (mycelial phase, broth filtrate) was the most active antigen in the precipitin tests and against hyperimmune sera in the complement fixation test. Whole yeast phase organisms as antigen gave the highest complement fixing titers with sera from infected animals. In addition, the particulate antigens of either phase were the only ones which reacted with sera from chronically infected rabbits. Cross-absorption tests performed by these authors suggested that at least one additional antigenic property was present in the whole yeast or mycelial cell than in the culture medium or ground-cell filtrates.

The first extensive use of serological test for histoplasmosis with human sera was by Furcolow, Bunnell, and Tennenberg,[31] employing a test devised by Tennenberg and Howell[32] consisting of an adaption of the Kolmer complement fixation test with histoplasmin as antigen. A group of 300 human sera was tested, and the fourteen positive reactors (++++) were limited to six cases of proved histoplasmosis, two recent histoplasmin skin test converters, and six histoplasmin-positive, tuberculin-negative persons with unhealed pulmonary lesions. Twenty ''doubtful'' (++ and +++) reactors were found throughout the 300 sera, but to a significantly lower per cent in the ''control'' group than in the groups enumerated above.

Saslaw and Campbell have also worked with the complement fixation test in histoplasmosis. They employed yeast-phase antigens, first whole washed heat-killed yeast cells,[33] and later an extract of ground yeast cells.[34] They found both antigens equally satisfactory in regard to sensitivity, but preferred the ground antigen because of greater stability for storage. These authors felt, after studies with several strains of *H. capsulatum*, that all made equally satisfactory antigens.

By hyperimmunizing rabbits to the other systemic fungi, Saslaw and Campbell found that cross reactions in the complement fixation test for histoplasmosis occurred only with blastomycosis. This finding was similar to that of Tennenberg and Howell[32] with guinea pigs infected with various fungi. Salvin[35, 36] has recently reported comprehensive studies on the serological relationships of fungi. These studies confirm the rather extensive cross reaction between blastomycosis and histoplasmosis, and in addition suggested the possibility of some lesser cross reactions with coccidioidomycosis and moniliasis. All these authors agreed that reactions with homologous sera were more intense.

In adition to the complement fixation test Saslaw and Campbell[38] developed a collodion agglutination test[37] using histoplasmin to sensitize the collodion particles. They employed both these serological tests for histoplasmosis in approximately 900 human sera. Included in the study were sixteen cases of proved histoplasmosis. ''Positive'' results (sera dilution of 1:40 or greater) were obtained in only four of these cases and in one of them only in the collodion agglutination test. The authors pointed out that the thirteen proved cases failing to react or reacting in low titer in the complement fixation test were late in the course of a fatal infection or had been chronically ill at least eight months. In addition, positive results were obtained in two children who were thought clinically to have histoplasmosis. Of interest were these authors' findings in a group of ''normal'' ambulatory persons with known skin-test reactions to histoplasmin. In 230 histoplasmin-positive persons, sera from 13.5 per cent were found to react at titers of from 1:5 to 1:40 in the complement fixation test, while sera from 4.9 per cent of 323 skin-test negative individuals reacted with the highest titer being 1:20. In the collodion agglutination test, ''weak (+) and (++) reactions'' were found in approximately 25 per cent of this normal group without significant variation between skin-test positive and negative groups. No other reports of broad experience with serological tests for histoplasmosis are available, although their use has been mentioned by several authors.[24, 27, 39, 40]

An extensive skin testing program of University of Chicago students had been going on for several years.[41] This program plus the facilities of the University Clinics, which draws patients from all over the midwest, offered an opportunity to study the usefulness of the complement fixation reaction in the study of histoplasmosis.

MATERIALS AND METHODS

Source of Sera.—Sera from 2,000 persons were tested. One thousand of these were taken from serological sera submitted routinely on University of Chicago Clinics admissions. Four hundred fifty-six represent sera submitted for influenza antibody determination over a two and one-half year period. Since most of these later sera were from University of Chicago students, information regarding skin test sensitivity was available on the majority.[41] Two hundred fifty-six sera were obtained from apparently healthy histoplasmin-positive students in Southern Illinois colleges during a skin test survey.[46] Sera from a total of 109 individuals were referred because of some type of pulmonary pathology. These sera came not only from the University Clinics, but from all over the midwest, with one group of forty-two sera from the Macon County Tuberculosis Sanitarium, Decatur, Ill.* Ninety-five sera were obtained from University of Chicago students during routine physical examination without regard for skin test sensitivity. The remaining eighty-four sera came mostly from patients of the University Clinics and University Health Service, and were referred for a variety of reasons.

Procedure.—The complement fixation test of Kent and Rein,[42] as modified for use with *H. capsulatum* by Saslaw and Campbell,[33] was adapted for these studies. As used on the sera in this report, the test consisted of adding to serological tubes in order: 0.2 ml. of the serum dilution, 0.2 ml. containing three 50 per cent units of complement, and 0.2 ml. of the standard dilution of antigen. The tubes were then incubated at 4° C. for eighteen hours. After addition of 0.2 ml. of 2 per cent washed sheep erythrocytes sensitized with 0.2 ml. of the optimal ambocepter dilution, the tubes were incubated at 37° C. for thirty minutes. These tubes were then centrifuged and read against a series of freshly prepared standards of hemolysis. Controls of each serum, as well as antigen, complement, and sensitized cells, were included in each test. The only variable in the test was the serum dilution. Twofold dilutions beginning with 1:5 of inactivated serum were used, and the results of the test were expressed in terms of the serum dilution. A serum was considered positive to the highest dilution exhibiting less than 50 per cent hemolysis.

Complement and ambocepter were obtained commercially† and titered by the methods of Kent and co-workers,[43, 44] employing color standards as indicators of percentage hemolysis. Color standards were prepared by mixing whole and laked cells previously brought to isotonic condition. Tubes representing each 5 per cent increment from 0 to 100 per cent hemolysis were prepared. All dilutions were made with phosphate buffered saline at 7.4 pH.[45]

Choice of Antigen.—The variety of preparations of *H. capsulatum* used as antigen in complement fixation tests have been mentioned above. At the initiation of these studies, preliminary evaluation was made of three preparations (whole yeast cell, ground yeast cell supernatant,[34] and histoplasmin) for their suitability as antigen in the above-outlined test. The whole yeast cell material consistently proved to be the most sensitive antigen, with sera from both hyperimmunized rabbits and two human cases of proved histoplasmosis. This antigen was prepared by scraping fresh yeast growth (Strain C1066) from the surface of enriched blood agar plates and heat killing at 57° C. for four hours. It was employed as antigen throughout the studies. Because of a tendency to become unsatisfactory after prolonged icebox storage, a fresh lot was prepared every six to eight weeks. Each new lot was titered against positive sera. The ideal dilution (from packed cells) varied from 1:200 to 1:800.

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On subsequent occasions, further studies of antigenic material were made employing new preparations of ground yeast cells and different lots of histoplasmin* tested against various positive sera. Table I presents an example of the three antigenic materials being titered at the same time against a highly positive human sera. The whole yeast cell preparation showed greater sensitivity with all the "high" titer human sera discovered in this study.

RESULTS

Table II presents a tabulation of the results obtained in testing the sera of 2,000 persons. For the purpose of this tabulation when more than one serum

TABLE I. EXAMPLES OF ANTIGEN TITRATIONS IN THE COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS WITH THREE TYPES OF ANTIGEN AGAINST A SINGLE HIGH TITER POSITIVE HUMAN SERUM PERFORMED AT ONE TIME

ANTIGEN		SERUM DILUTION							ANTIGEN
TYPE	DILUTION	1:10	1:20	1:40	1:80	1:160	1:320	1:640	CONTROL
Whole yeast cell No. 25	1:200	0	0	0	0	0	0	50	50
	1:400	0	0	0	0	20	50	98	98
	1:800*	0	0	0	0	30	90	100	100
	1:1600	0	0	0	10	40	100	100	100
	1:3200	0	0	10	30	90	100	100	100
Histoplasmin	1:50	0	0	20	80	90	95	100	90
	1:100	0	0	60	95	100	100	100	98
	1:200	10	30	95	98	100	100	100	100
	1:400	50	100	100	100	100	100	100	100
	1:800	90	100	100	100	100	100	100	100
Ground yeast cell No. 5	1:2	0	0	0	50	60	60	50	5
	1:4	0	40	90	98	100	100	100	95
	1:8	0	90	98	100	100	100	100	100
	1:16	90	98	100	100	100	100	100	100
	1:32	100	100	100	100	100	100	100	100

*Dilution chosen for use.

TABLE II. RESULTS OF COMPLEMENT FIXATION TESTS FOR HISTOPLASMOSIS ON SERA FROM 2,000 PERSONS GROUPED BY CLINICAL DATA SUGGESTING THE LIKELIHOOD OF ACTIVE *Histoplasma* INFECTION

	NEG.	1:5	1:10	1:20	1:40	1:80	1:160	1:320	TOTAL	PER CENT REACTORS
Proved histoplasmosis				2		1	3	1	7	100
Pulmonary infiltrates H.+ T.°	13	4	7	4	2		1	1	32	59
Histoplasmin converters	12	4	1						17	29
Pulmonary infiltrates H.+ T.+	39	2		1	1				43	9
Benign respiratory disease H.+	71	2	4	1					78	9
Miscellaneous H.+	43		3						46	7
Southern Illinois students H.+	231	11	11	3					256	10
Miscellaneous H.?	219	1		1					221	0.9
Routine serological sera H.?	993	6	1						1000	0.7
Histoplasmin negative	299	1							300	0.3
Total	1920	31	27	12	3	1	4	2	2000	

H.+ Histoplasmin positive.
T.° Tuberculin negative.
H.? Histoplasmin unknown.

*Kindly supplied by Dr. J. C. Peterson, Vanderbilt University, and the U.S.P.H. Tuberculosis Control Division.

was tested from the same individual, the highest titer result is used. The persons are grouped by the available data which would determine the probability of the presence of an active *Histoplasma* infection. The groups are placed from top to bottom in descending order of that probability with the culturally proved cases of histoplasmosis at the top. Persons with nontuberculous pulmonary infiltrates who reacted to histoplasmin and histoplasmin converters (within one year) were considered the most likely to have active histoplasmosis. The group with pulmonary infiltrates reacting to both tuberculin and histoplasmin was heavily overbalanced with thirty-three known active cases of tuberculosis. The group labeled "benign respiratory disease" represent the sera submitted for influenza study.

It can be readily seen from Table II that the eighty reacting sera concentrate in the upper groups with only one of 300 persons who were skin-test negative reacting, and that at the lowest (1:5) dilution. Six 1:5 and one 1:10 were the only reactions in the 1,000 Clinics admissions. The eight reactions above 1:40 are found only in the proved and most likely active infection groups. Sera from all of the cases of culturally proved histoplasmosis reacted in the complement fixation test, with five reacting in high titer. The two proved cases with sera reacting at 1:20 were both elderly persons near death from generalized histoplasmosis. Other sera obtained on these patients prior to death reacted in even lower titer.

Table III is a condensation of Table II, showing in percentages the occurrence of "high" and "low" titer reactions. The division of "high" and "low" at 1:40 sera dilutions is entirely arbitrary. This table shows that high

TABLE III. THE PERCENTAGE OCCURRENCE OF REACTIONS IN THE COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS AMONG INDIVIDUALS GROUPED ACCORDING TO THE LIKELIHOOD OF THE PRESENCE OF ACTIVE *Histoplasma* INFECTIONS

		NEGATIVE	LOW (1:40 AND BELOW)	HIGH (ABOVE 1:40)
Culturally proved histoplasmosis	No.	0/7	2/7	5/7
	Per cent	0	29	71
Suspected* active histoplasmosis	No.	25/49	22/49	2/49
	Per cent	51	45	4
Other histoplasmin positive	No.	383/421	38/421	0/422
	Per cent	91	9	0
All histoplasmin negative	No.	299/300	1/300	0/300
	Per cent	99.7	0.3	0

*H.+ T°, pulmonary infiltrates and histoplasmin converters.

titer reactions occurred frequently (71 per cent) in those patients with proved histoplasmosis, but only infrequently (4 per cent) in patients suspected of having active disease but from whom cultural proof was not obtained, and not at all in other groups. Low titer reactions occurred in an additional 45 per cent of the suspected cases, bringing to near 50 the percentage of reactors in this group. Nine per cent reactions were found in the group of histoplasmin-positive persons in whom T° infiltrates or recent conversion was not demonstrated.

TABLE IV. RESULTS OF COMPLEMENT FIXATION TESTS FOR HISTOPLASMOSIS WHERE MORE THAN ONE WAS AVAILABLE ON THE SAME PERSON OVER AT LEAST A THREE-MONTH INTERVAL

	MONTHS								
	0	1	2	3	4	5	6	7	8
Case 1	1:320	1:160	1:80	1:40	–	Neg.	–	Neg.	
Case 2	1:160	1:80	–	1:80	1:40	–	–	–	1:80
Case 3	1:160	1:80	1:40	1:20	–	1:20	–	Neg.	
Case 4	1:160	1:5	Neg.	–	Neg.	–	Neg.	–	Neg.
Case 5	1:80	–	–	–	–	1:40			
Case 6	1:20	1:40	–	–	1:5	1:5	–	–	Neg.
Case 7	1:20	1:20	1:10	1:10	–	–	Neg.		
Case 8	1:20	1:20	–	–	Neg.				
Case 9	1:20	1:20	1:10	Neg. (died)					
Case 10	1:20	–	–	–	–	Neg.			
Case 11	1:10	–	–	–	–	Neg.			
Case 12	1:10	–	–	Neg.					
Case 13	1:10	1:5	Neg.	Neg.					
Case 14	1:5	–	–	1:5					
Case 15	Neg.	–	–	1:5	–	–	Neg.		

There were fifteen persons showing a positive reaction on whom more than one serum was tested over at least a three-month period. Table IV shows the findings in these persons over as long as sera were available. It can be seen that positive reactions had disappeared in twelve of the individuals within eight months or less after the initial blood specimen. The two high titer sera which persisted over five and eight months, respectively, were from patients suffering from chronic pulmonary disease proved by culture to be histoplasmosis. In both of these persons the onset of their illness predated the initial sera examination by over six months. Slow clinical improvements continued in both patients, with Case 2 now considered well after three years and Case 3 after two years still showing pulmonary disease and a positive complement fixation test.

In only two individuals was a rise in titer demonstrated. Case 6 with an initial titer of 1:20 which rose to 1:40 in a month and then gradually fell to negative in seven months was a healthy university student who was found to have a "fresh-appearing" infiltrate on admission x-ray examination. This student never had symptoms, and maintained ordinary activity. Follow-up chest x-rays over a two-year period showed slow healing by fibrosis. The rise from negative to 1:5 and subsequent fall to negative was demonstrated coincidentally with histoplasmin skin conversion in a laboratory worker after exposure to the mycelial phase of *Histoplasma capsulatum*.

The majority of persons in Table IV showed a rather rapid fall in titer to negative. However, the time of the first serum has a variable and frequently unknown relation to the onset of the infection for most of the fifteen individuals. One fortunate exception is five members of the "E" family. These include the remaining three so far undiscussed high titer sera shown in Table IV. These three had onset of symptoms on or about Sept. 28, 1950, and epidemiological evidence[16] suggests that they were infected simultaneously two to three weeks previously. In addition, two other members of the family

TABLE V. COMPLEMENT FIXING ANTIBODIES AGAINST *H. capsulatum*. RESULTS IN SERA FROM MEMBERS OF THE "E" FAMILY IN WHOM THE ONSET OF ILLNESS WAS SEPT. 28, 1950

DATE	A. E., SR. 49 YEARS	E. E. 21 YEARS	A. E., JR. 5 YEARS	AL E. 19 YEARS	M. E. 17 YEARS
11/ 2/50	1:160				
11/10/50		1:320	1:160		
11/18/50	1:10			1:10	
11/29/50	1:5				
12/ 1/50		1:160	1:80		1:20
12/15/50	1:5				
1/10/51	Neg.	1:80	1:40		
2/24/51	Neg.	1:40	1:20		
4/23/51	Neg.	Neg.	1:20	Neg.	Neg.
6/23/51	Neg.	Neg.	Neg.	Neg.	Neg.

had asymptomatic pulmonary infiltrates associated with lower titer reactions in the complement fixation test for histoplasmosis. Table V presents in detail the results obtained on sera from this family.

The precipitous fall in titer of A. E., Sr.'s sera coincided with the onset of the critical period of his illness which was characterized by high fever and extension of pulmonary infiltrates. The patient gradually recovered from this illness over the succeeding months, but his sera never again reacted in the complement fixation test. The other members of the family either never had symptoms or had been clinically well for at least a month when their sera was first examined. The gradual and regular fall in titer in these persons coincided with a gradual clearing and healing by calcification of their pulmonary lesions.

The clinical outcome of the two cases with high titer serology whom it was not possible to follow over any period of time was as follows: the patient whose sera reacted at the 1:320 dilution was a 10-year-old boy who died a week after the blood sample of generalized histoplasmosis complicated by toxicity of ethyl vanillate. The patient who reacted at 1:160 was an elderly man who had suffered from a chronic mucomembranous *Histoplasma* lesion for over two years. This lesion slowly healed over the year and one-half after the blood specimen was obtained. There was never any sign of generalized disease.

REPRODUCIBILITY OF RESULTS

With each test performed, at least one positive control serum was run in addition to the regular antigen and sera controls. The test was considered acceptable if adequate sensitivity was demonstrated in the known positive serum without occurrence of anti-complementary activity in the controls. No serum in this study was discarded as negative unless it failed to react in two acceptable tests. All positively reacting sera were tested at least twice, and usually many more times as positive controls and in antigen titrations. The amount of variability observed on the same serum did not exceed one twofold dilution. The most frequent variability was present in the results with high titer sera. Table VI presents the results with one such serum in fifteen consecutive tests in which this serum was employed as a positive control.

TABLE VI. COMPLEMENT FIXATION TESTS FOR HISTOPLASMOSIS ON ONE SERUM (L. D., 10/12/50) PERFORMED ON DIFFERENT DATES

DATE	ANTIGEN AND DILUTION	SERA DILUTIONS					
		1:20	1:40	1:80	1:160	1:320	1:640
10/13/50	W#20 (1:200)	0	0	0	0	90	100
10/18/50	W#21 (1:800)	0	0	0	0	30	90
10/31/50	W#21 (1:300)	0	0	10*	60	100	100
11/ 3/50	W#22 (1:300)	0	0	0	0	30	90
2/25/51	W#24 (1:300)	0	0	0	25	75	98
3/ 4/51	W#24 (1:300)	0	0	0	0	40	98
3/18/51	W#24 (1:400)	0	0	0	0	35	90
4/15/51	W#24 (1:400)	0	0	0	0	0	50
5/22/51	W#25 (1:800)	0	0	0	0	0	90
5/24/51	W#25 (1:600)	0	0	0	10	10	55
6/ 2/51	W#25 (1:800)	0	0	0	0	90	100
6/18/51	W#25 (1:600)	0	0	0	40	90	100
6/19/51	W#25 (1:600)	0	0	0	30	90	95
6/22/51	W#26 (1:600)	0	0	0	30	60	90
6/28/51	W#26 (1:800)	0	0	0	20	30	90

*Note: Test of 10/31/50 discarded as unsatisfactory because it failed to demonstrate adequate sensitivity.

DISCUSSION

In order to evaluate the usefulness of the complement fixation reaction in determining active *Histoplasma* infection, both the specificity and the sensitivity of the test must be considered. The rare occurrence of a positive reaction in persons in whom active infection seemed unlikely supports the specificity of the test. It is of interest that the only serological reaction that occurred in a group of 300 histoplasmin skin test negative individuals was in a person on whom the only skin test available had been performed six months prior to submission of the blood sample. The patient had suffered from a mild febrile illness, and the blood had been submitted for influenza studies. It was not possible to obtain repeat skin tests. The occurrence of but seven reactions in 1,000 consecutive Clinics admissions further supports the specificity of the test as used in this study. Since all these reactions were in the lowest two dilutions, it seems that if rare nonspecific reactions occur they will be confined to the low dilutions.

The tabulation of results as presented in Table II offers evidence of the sensitivity of the test. A regular relationship is found between the clinical probability of active *Histoplasma* infections and the occurrence of positive results in the complement fixation test. One possible exception to this statement would be the group of histoplasmin-positive students in which 10 per cent reacted. This rather high percentage of reactions in an apparently healthy group could be explained by an unexpected number of recent skin test "converters." A high rate of conversion would be made possible by a sufficient number of skin-test negative individuals attending these schools in Southern Illinois, an area where an unusually high prevalence of histoplasmin reactions occurs.[46] Support for this explanation was obtained by review of the residence histories of twenty-four of the twenty-five students with positive sera. It was found that twelve of them had lived most of their lives in areas

of low prevalence of histoplasmin reactions (mostly metropolitan Chicago and New York City).

The finding of positive reactions in all cases of proved histoplasmosis appears to be dependent on the phase of the disease occurring at the time the blood sample is obtained. The fall in titer of two proved cases to negative one just prior to death and the other during obvious active disease (A. E., Sr.) demonstrates that negative reactions can at times occur in proved cases.

Correlation of height of titer and the clinical significance of the infection is difficult. The seven "high" titer reactions in this study were from patients with histoplasmosis proved either culturally or by very strong epidemiologic evidence. Although each of these persons suffered a definite illness, the duration and severity varied greatly. The occurrence of symptomatology in connection with the lower titer reactions was inconstant. Most persons suffered no illness at all, while many had short febrile reactions. Only rarely was a severe illness associated with a "low" titer reaction. It would seem justified to conclude that in general the higher the titer of a positive reaction, the more likely a clinically significant *Histoplasma* infection.

The occurrence of the "low" titer reactions in asymptomatic persons with pulmonary infiltrates, recent skin test conversion, or laboratory exposure to the fungus, adds further support to the present concept of histoplasmosis as a common benign infection.

The definite prognostic significance attributed to serological tests in other generalized mycosis[25, 26] would not appear to hold true for histoplasmosis. While the number of proved cases examined in this study is very small, there was considerable divergence of the serological response and the clinical courses and outcome.

The complement fixation test for histoplasmosis has proved to be of definite usefulness. The cultural proof of two of the cases came as a direct result of finding positive serology. The speed with which results are obtained allowed a fruitful epidemiologic investigation[16] to be undertaken three weeks prior to cultural identification of the infection. The test has rendered aid in the differential diagnosis of pulmonary lesions, allowing the diagnosis of probable tuberculosis to be avoided in several cases where positive complement fixation tests had been obtained. The subsequent course of these patients has justified the use of a less conservative approach to therapy than would have been necessary as long as tuberculosis was suspected.

From this study, the opinion has evolved that the test is a valuable aid in diagnostic studies. The ease with which sera may be sent to a central laboratory and the rapidity with which results can be obtained are definite advantages. When a specific therapy for this infection has been found, this speed with which a presumptive diagnosis can be made may assume much greater importance.

It is necessary to appreciate the limitations of the test when interpreting results. The cross reaction with blastomycosis, while not studied here because of the failure to find any patients with suspected *Blastomyces* infection, is well

established.[32, 34, 36] This drawback can be overcome by concurrent testing with *Blastomyces* antigen. The possibility of some patients with active histoplasmosis failing to react in the test has been pointed out. Although this would appear to be an uncommon occurrence from the results of this study, it has occurred more frequently with other types of antigen.[38] The rapidity of development of complement fixing antibodies after infection cannot be determined from this study. Certainly they were well established in the "E" family when first examined about two months after exposure to the fungus. In general, the possibility of false negative reactions can be minimized by obtaining serial blood specimens. Serial examinations also allow more accurate estimation of the significance of titer.

SUMMARY

A complement fixation test for histoplasmosis employing a whole yeast cell preparation as antigen was performed on sera from 2,000 individuals. Seven patients with culturally proved histoplasmosis were encountered. Sera from each of these patients reacted in the test, five at serum dilutions greater than 1:40, and the remaining two at 1:20 dilutions. Only two other reactions above 1:40 were found. These were in two sons of a proved case where there was strong epidemiologic evidence of active *Histoplasma* infection. Of the forty-nine persons suspected of having active *Histoplasma* infections on the basis of recent histoplasmin skin test conversion or pulmonary infiltrates along with positive histoplasmin and negative tuberculin skin tests, 50 per cent reacted in the complement fixation test. Conversely, among 300 persons in whom active *Histoplasma* infections seemed unlikely on the basis of negative histoplasmin skin tests, only one reaction occurred and that at the lowest (1:5) dilution employed. It was concluded that the complement fixation reaction has a definite usefulness in diagnostic studies for histoplasmosis. Some of the advantages and the limitations of the test are discussed.

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THE INFLUENCE OF IN VITRO HEMOGLOBIN MODIFICATION ON HEMOGLOBINURIC NEPHROSIS IN RABBITS

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NORMAL animals are capable of either storing or excreting hemoglobin following large (1.5 to 2.9 Gm. per kilogram) or massive (3.0 to 6.0 Gm. per kilogram) intravenous injections. Well-fed dogs[1, 2] or rabbits[3, 4] which have access to water tolerate multiple intravenous oxyhemoglobin injections without apparent renal injury. Localization of oxyhemoglobin or its derivatives in the distal convoluted tubules of animals has been accomplished only when the intravenous hemoglobin injections were combined with injections of arsine,[5] of histamine,[6] or preceded by injections of sodium tartrate,[3] by unilateral nephrectomy and renal artery ligation,[3, 7] or by the deprivation of food and water for four to five days.[4] It has been demonstrated and confirmed that the production of tubular injury prior to the hemoglobinemia favors the accumulation of oxyhemoglobin or its derivatives in the kidneys. Experimental studies have also established the fact that oxyhemoglobin is relatively nontoxic to animals having adequate reserves of body fluid and sufficient sodium to maintain an alkaline urine and normal kidneys.[3, 4, 8] Bywaters[9] has inferred that myoglobin is toxic. His experiments, however, have not excluded other factors of a toxic nature which are inherent in the crush syndrome. In spite of the prevailing opinion that oxyhemoglobin is relatively nontoxic, it has been suggested that this pigment may on occasion exert some influence on the renal tubules.[2, 4, 10, 12] It is not apparent, however, whether the pathologic and physiologic changes are in response to the presence of abnormal concentrations of electrolytes and oxyhemoglobin in the tubules or physiochemical changes which have occurred in the oxyhemoglobin molecule.

Studies in which some physicochemical properties of oxyhemoglobin or its derivatives are compared with the biologic response may prove helpful in elucidating the role of hemoglobin in this syndrome. Bing[13] has shown that methemoglobin is nephrotoxic when injected into dogs with aciduria. That this protein should exert a toxic effect is noteworthy, since it has been demonstrated that methemoglobin is readily reduced to oxyhemoglobin in the body following intravenous injections.[2] In view of the difference in biologic response to methemoglobin and oxyhemoglobin, and the knowledge that these substances are complex proteins which may undergo modification either before or after intravenous injection, further observations on modified hemoglobin are warranted. Alterations in oxyhemoglobin have been shown to occur following the preparation of the dry compound,[14] exposure of solution to room temperature,[15]

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variation in pH,[16, 17] or the addition of chemicals.[18, 19] Some of these known methods were employed to modify the hemoglobin. Thereafter, one or more physiochemical properties of the modified oxyhemoglobin were ascertained, and the response to intravenous injection was determined.

METHOD

New Zealand white rabbits of both sexes weighing from 2.0 to 3.7 kilograms were used in this study. The animals were kept in individual metabolism cages during the period of observation. Water and food consumption, as well as urine excretion, were noted for seven days prior to and up to ten days after the intravenous injections of hemoglobin. The urine was analyzed for its volume, specific gravity, and pH. Since acid urine may predispose to the retention of pigment casts in the renal tubules,[3, 4, 20] these rabbits were fed different diets. Some animals were given an acid diet, whereas others received an alkaline diet of rabbit pellets.* The effect of intravenous injection was determined for oxyhemoglobin kept at 4° C. up to 330 days, acidified and neutralized hemoglobin and oxyhemoglobin converted to methemoglobin. The hemoglobin was prepared as in previous studies.[4, 11, 21] Following the second washing with physiological saline and centrifugation at 3,000 r.p.m. for thirty minutes, hemolysis was accomplished by adding an equal quantity of distilled water to the packed erythrocytes. Ether, equivalent to 10 per cent of the combined volume of cells and water was then added, and this mixture was vigorously shaken for ten minutes. The ether accelerated the hemolysis and rendered the solution bacteriostatic. The mixture of stroma, oxyhemoglobin, and ether was stored at 4° C. for variable periods of time before injecting into the first group of animals. The hemoglobin was separated from the stroma on the day of injection. This was done by centrifugation at 10,000 to 13,000 r.p.m. for thirty minutes. Following centrifugation, the oxyhemoglobin solution was finally passed through a fritted glass filter with the aid of vacuum to insure the removal of any remaining solid particles. Centrifugation and filtration also eliminated approximately 90 per cent of the ether by evaporation. The solution which was injected into a second group of animals was acidified with 0.1N HCl until the pH was depressed to 4.95-5.05. The addition of acid with constant agitation resulted in the formation of coarse aggregates and changes in oxyhemoglobin color. Immediately thereafter, the pH was returned to its original reading of 7.4 by the addition of 0.1N NaOH. The solution was then centrifuged and subsequently passed through a filter. This modified hemoglobin was injected on the same day it was prepared. The methemoglobin which was injected into the third group of rabbits was prepared by mixing 2 ml. of 1 per cent sodium nitrite with 5 ml. of oxyhemoglobin from which the stroma and ether had been previously removed.

The oxyhemoglobin concentration and its absorption characteristics were ascertained in 1:100 dilution of 0.1 per cent Na_2CO_3 in a Beckman Quartz Spectrophotometer. Since the characteristic absorption of oxyhemoglobin[22] was modified by acidification and neutralization, added studies were done on this sample. The per cent of active and inactive hemoglobin was determined by the carbon monoxide method.[23] The same solution was also analyzed for its methemoglobin concentration by a photometric method.[24] The plasma nonprotein nitrogen (NPN)[25] was done during the control period and on the fifth day after the intravenous hemoglobin injections. All rabbits were autopsied on the eighth day except for nine in the first group which did not manifest any changes in urine excretion. Following gross examination, samples of tissue were fixed in 10 per cent formalin. After embedding in paraffin, sections were stained with hematoxylin and eosin (H and E) for microscopic examination. The pathologic changes which may be observed in experimental hemoglobinuric nephrosis have been repeatedly described.[4, 11, 21] Since the purpose of this study is to determine why hemoglobin is retained in the renal tubules, special emphasis is placed on the pigment casts. This emphasis is based on the hypothesis that any factors which favor the

*Master mix rabbit pellets (McMillen Feed Mills, Division of Central Soya Company, Inc., Fort Wayne, Ind.).

accumulation of pigment casts in the distal convoluted tubules also probably contribute to oliguria, anuria, and uremia. Pigment cast accumulation is expressed as a total number of casts observed in ten random low-power fields.

RESULTS

1. *Physiochemical Studies of the Different Hemoglobin Solutions.*—Three or four separately prepared hemoglobin solutions were analyzed and injected into each group of rabbits. The absorption spectra shown in Fig. 1 are of three representative samples of the different preparations which were subsequently injected.

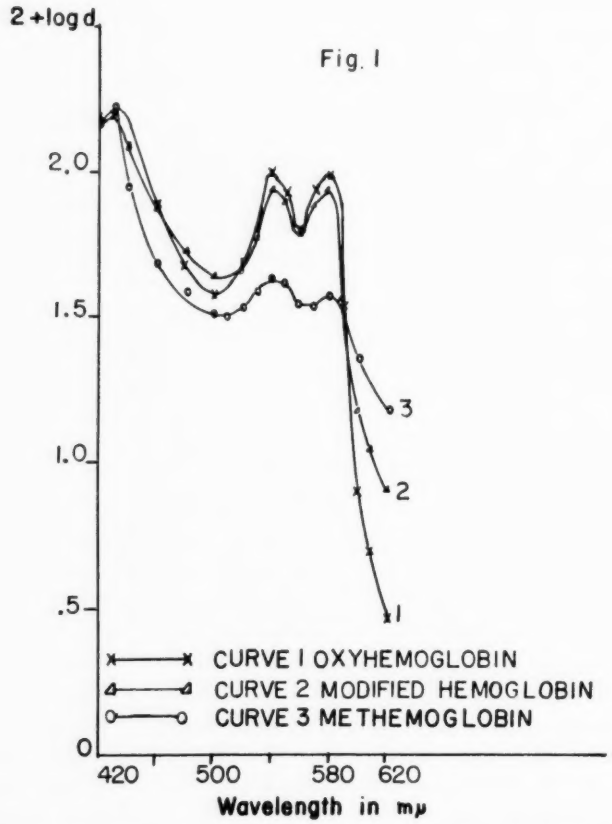

Fig. 1.—Absorption spectra of the different hemoglobin solutions.

Prior to absorption studies the control (Curve 1) and treated solution (Curve 2) were diluted so that the hemoglobin concentrations were equal. Examination of Curve 2, which represents acidified and neutralized hemoglobin, shows that some alteration has occurred in the protein. There is decreased absorption at 540 and 580 mμ; the curve also crosses the untreated control at 460, 516, and 590 mμ. Aging the oxyhemoglobin up to forty-nine days exerts no effect on the absorption spectrum; therefore, Curve 1 is also representative

of the absorption spectra of the oxyhemoglobin preparations which were injected into twelve rabbits in the first group. The hemoglobin which was permitted to stand for 330 days, however, had decreased absorption at 540 and 580 mμ. Curve 3 shows that the addition of sodium nitrite converts the oxyhemoglobin to methemoglobin. Since minor changes were effected by acidification and neutralization, added studies were done on each of these samples. Observations made on one such preparation are shown in Table I. The carbon monoxide method for estimation of active and inactive hemoglobin as well as the methemoglobin determination also indicates that acidification and neutralization produce changes.

The oxyhemoglobin concentration of control and treated solutions diluted to the same concentration when analyzed by spectrophotometric absorption at 540 mμ did not correspond. The differences in concentration were attributed to decreased absorption of the treated solution at 540 mμ. There is a closer agreement between the concentration values when the spectrophotometric absorption of oxyhemoglobin is compared to the total hemoglobin value obtained with carbon monoxide. Of 8.75 Gm. of oxyhemoglobin, 2.07 Gm. were inactivated by acidification and neutralization. The formation of methemoglobin could account for the differences in absorption observed in Curve 2. The concentration of carbon monoxide inactive hemoglobin is somewhat less than that of methemoglobin. Similar differences were observed in the analyses of other samples.

2. *Biologic Response to the Different Hemoglobin Solutions.*—Thirty of thirty-eight rabbits which were studied are shown in Table II. The animals were injected with oxyhemoglobin which was permitted to age (Type A), acidified and neutralized oxyhemoglobin (Type B), and methemoglobin (Type C). Each animal received a total of 1 gm. per kilogram in one day except for two rabbits which were given 330-day-old hemoglobin, in which case 0.5 Gm. per kilogram was administered. A diet of ground oats with 1 per cent NaCl, which was fed to the first group, is known to be deficient in protein, vitamins, and minerals.[26] To establish an aciduria without a dietary deficiency the second and third groups of animals were fed twelve parts soybean meal, fifty-five parts ground oats, and twelve parts cerelose which was supplemented with the necessary vitamins and minerals. The acid diets depressed the urine pH to levels of 5.0 to 5.6 within two weeks' time. Animals fed rabbit pellets excreted a urine in which the pH fluctuated from 7.5 to 8.9.

TABLE I. PIGMENT CONCENTRATIONS OF CONTROL AND TREATED SOLUTIONS DETERMINED BY DIFFERENT METHODS

METHOD	CONTROL HEMOGLOBIN (GM.)		ACIDIFIED AND NEUTRALIZED HEMOGLOBIN (GM.)		
	TOTAL	INACTIVE	TOTAL	ACTIVE	INACTIVE
Oxyhemoglobin absorption at 545 mμ	8.65		8.10		
Carbon monoxide method		*	8.75	5.68	2.07
Methemoglobin absorption at 800 mμ		0.50			2.52

*In analyses of other control preparations the inactive hemoglobin concentration seldom exceeded 5 per cent.

Five rabbits (No. 1 to 5) were injected with hemoglobin aged four to six days to exclude the possibility of a spontaneous change in the solution during its preparation. These animals served as controls for the rabbits which were injected with acidified and neutralized hemoglobin (No. 11 to 20). Nine other animals, five of which are shown in the table, received oxyhemoglobin which varied in age from 30 to 330 days. Four rabbits which received thirty-day-old oxyhemoglobin have not been included in Table II. The urine excretion was not significantly diminished irrespective of the acid or alkaline diets in twelve rabbits injected with a total of 1 Gm. per kilogram. In one or two days after the hemoglobin or its derivatives were excreted, the urine volume, the specific gravity, and the pH were comparable to values observed during the control period. A diminution in food consumption which was encountered in previous studies in rabbits with renal injury did not occur. In five rabbits (No. 6 to 10) food and water deprivation for one or two days exerted no apparent effect. Nothing unusual was observed in two rabbits (9 and 10) which received 0.5 Gm.

TABLE II. RESPONSE OF RABBITS TO DIFFERENT HEMOGLOBIN SOLUTIONS

RABBIT NUMBER	DIET	FOOD & WATER WITHHELD (DAYS)	AGE OF HEMOGLOBIN (DAYS)	TYPE OF HEMOGLOBIN INJECTED	URINE EXCRETION	PLASMA NPN 5TH DAY (MG. %)	COMBINED KIDNEY WEIGHT (GM.)	TOTAL† PIGMENT CASTS
1	Pellets	0	4	A	Normal	26	19.0	0
2	Oats	0	4	A	Normal	25	12.9	66
3	Oats	0	6	A	Normal	32	16.6	15
4	Pellets	0	6	A	Normal	36	12.5	5
5	Oats	0	6	A	Normal	44	16.0	11
6	Oats	1	49	A	Normal			
7	Oats	1	49	A	Normal			
8	Oats	1	49	A	Normal			
9	Oats	2	330*	A	Normal			
10	Pellets	2	330*	A	Normal			
11	Pellets	0	4	B	Normal	24	16.0	0
12	Pellets	2	4	B	Normal	22	21.2	25
13	Pellets	2	6	B	Normal	25	16.3	0
14	Pellets	2	6	B	Died first day			17
15	Pellets	0	6	B	Oliguria anorexia	72	26.5	170
16	Soybean	0	4	B	Normal anorexia	28	12.3	0
17	Soybean	2	4	B	Normal	28	14.5	58
18	Soybean	2	6	B	Normal	28	12.5	46
19	Soybean	2	6	B	Normal	40	16.5	13
20	Soybean	0	6	B	Normal	44	22.3	38
21	Pellets	0	4	C	Normal		13.6	2
22	Pellets	0	4	C	Normal	38	20.0	44
23	Pellets	0	6	C	Normal	22	22.4	23
24	Pellets	0	6	C	Normal	48	20.6	0
25	Pellets	0	6	C	Normal	90	19.1	0
26	Soybean	0	4	C	Normal	64	26.2	239
27	Soybean	0	4	C	Normal	34	20.8	333
28	Soybean	0	6	C	Oliguria	158	26.2	231
29	Soybean	0	6	C	Normal	82	23.9	168
30	Soybean	0	6	C	Normal	64	23.2	160

A = Oxyhemoglobin; B = modified hemoglobin; C = methemoglobin.

*Numbers nine and ten received 0.5 Gm. per kilogram, while the others were given 1 Gm. per kilogram.

†Total pigment casts observed in ten random low-power fields.

per kilogram of 330-day-old oxyhemoglobin. None of the animals in this group died. The plasma NPN values, the pigment cast counts, and kidney weights were not significantly elevated in rabbits in which such studies were done.

The oxyhemoglobin which was modified by acidification and neutralization was collected and prepared on three separate occasions. Four or more rabbits were studied at one time. The results in ten of fourteen such rabbits are included in Table II. Eight rabbits were fed an alkaline diet, whereas six were given an acid diet. Food and water were withheld for two days prior to intravenous injections in six animals (No. 12 to 14 and No. 17 to 19). One-half of the rabbits in this group developed symptoms following the second or third and last injection. Seven animals became so weak they could not stand until an elapse of from fifteen to forty-five minutes. The systolic blood pressure which was recorded at such times with Grant's ear cuff[27] dropped from 10 to 30 mm. Hg. One of the rabbits died within thirty minutes after the last injection. Two other rabbits stopped eating for several days. The urine excretion was temporarily diminished in No. 15. Unlike old oxyhemoglobin or the methemoglobin, the modified preparations produced an immediate toxic reaction. In spite of the immediate response, however, neither tubular degeneration nor appreciable numbers of pigment casts were encountered except in one rabbit (No. 15). In three rabbits (No. 12, 15, and 20) the kidneys were heavier at the time of autopsy. Significant renal damage as indicated by plasma NPN, combined kidney weight, and pigment cast count was encountered in one of fourteen rabbits (No. 15). It is not apparent whether diet exerted any effect on pigment cast accumulation in this group.

The results in ten rabbits injected with methemoglobin prepared by the addition of sodium nitrite are shown in Table II. The alkaline and acid diets were fed at least two weeks to ten rabbits prior to the injection of methemoglobin. These animals received a total of 100 to 160 mg. of $NaNO_2$ in divided doses at intervals of three to four hours. In control studies rabbits tolerated single intravenous injections of 70 to 100 mg. of $NaNO_2$ with no ill effects. The urine excretion following injections of methemoglobin was not significantly diminished except in one rabbit (No. 28). The NPN concentration exceeded 80 mg. per cent in three of ten rabbits (No. 25, 28 and 29). The increase in kidney weights and the number of pigment casts observed were the most significant findings in this group. The combined kidney weight exceeded 20 Gm. in seven of ten rabbits. It is of interest to note that appreciable increases in weight also occurred irrespective of the presence of pigment casts. The pigment cast counts were greater, however, in the kidneys of rabbits fed an acid diet (No. 26 to 30).

DISCUSSION

Oxyhemoglobin mixed with stroma and ether which is permitted to age at 4° C. up to forty-nine days reacts no differently after intravenous injections than solutions which are less than one week old. Aging of the oxyhemoglobin up to forty-nine days did not alter the absorption spectrum, whereas the solution which was kept for 330 days had decreased absorption at 540 and 580 mμ. Injections

of 0.5 Gm. per kilogram of the latter into two rabbits produced no toxic effects. Under the conditions of this experiment neither the withholding of water for one to two days nor the acid diets exerted any demonstrable effect.

When the oxyhemoglobin was acidified and neutralized prior to intravenous injection, it was possible to modify the protein. That an alteration had occurred is evidenced by changes in the absorption spectrum, reactions to carbon monoxide, and alterations in the methemoglobin concentration. This modified hemoglobin, unlike oxyhemoglobin which was permitted to age, was toxic since half of the animals receiving it were unable to stand after injection. In spite of the unquestionable immediate weakness, the blanching of ear veins, and appreciable decreases in systolic blood pressure, neither significant increases in kidney weight nor appreciable numbers of pigment casts were observed in the second group of rabbits. The observation of increased NPN, kidney weight, and pigment casts in one of fourteen rabbits suggests that some factor other than modification of hemoglobin is responsible.

In the last group of rabbits it was possible to demonstrate that methemoglobin which is prepared in vitro is retained to a greater degree in kidneys of animals which are fed an acid diet. This observation agrees with the findings of Bing, who demonstrated that NH_4Cl acidosis predisposes to the accumulation of pigment casts after intravenous methemoglobin injections. The methemoglobin in this study, however, was not as toxic to the rabbit as Bing has reported in the dog. This discrepancy in methemoglobin toxicity which has been encountered may be attributable to several factors. Bing administered NH_4Cl to produce acidosis in dogs prior to the intravenous injections of methemoglobin. Depending on the NH_4Cl dosage and the condition of the animal at the time, either a functional or anatomical tubular injury may have been produced by NH_4Cl administration. Salts that produce acidosis may be nephrotoxic since tubular degeneration with necrosis has been produced after administering NH_4Cl[28] and $CaCl_2$.[29] In view of this, it is not apparent whether the pigment cast accumulation in Bing's experiment is related to a tubular injury or the methemoglobin injections. The present studies favor the hypothesis that methemoglobin is responsible since antecedent tubular injury was not present in our animals. Sodium nitrite was used in this experiment, whereas Bing employed potassium ferricyanide to oxidize oxyhemoglobin to methemoglobin. Further investigation should determine whether the differences in methemoglobin toxicity are due to the oxidizing salts, an unexpected intolerance for methemoglobin in the dog, or the NH_4Cl administration.

The observations which have been made following methemoglobin injections raise several questions. The most important is whether the conditions of this experiment are artificial, having no bearing on the renal changes which may be observed in man. If methemoglobin is exerting an influence, then the associated urinary complexes capable of oxidizing oxyhemoglobin are important in the production of hemoglobinuric nephrosis. Since the methemoglobin was produced in vitro, it should be established whether similar renal changes will occur when an oxidizing solution and oxyhemoglobin are injected simultaneously by different routes.

Experimental attempts to produce hemoglobinuric nephrosis have on occasion been justifiably criticized as artificial.[1, 30] In spite of somewhat artificial conditions, however, it has been established that antecedent tubular injury, aciduria, and adequate reserves of body fluid exert a pronounced effect on the development of hemoglobinuric nephrosis. Artificiality can be ascribed to some experimental hemoglobinuric nephrosis studies because large amounts (1.5 to 2.9 Gm. per kilogram) of oxyhemoglobin were employed.[8, 11] Nevertheless, even in such experiments the important fact is emphasized that native oxyhemoglobin is relatively nontoxic to a normal animal. Studies which attempt to evaluate the role of oxyhemoglobin or its derivatives should restrict the dosage of intravenous hemoglobin to quantities comparable to those which are observed in man. Hemoglobinemia in human beings seldom exceeds a concentration of 1 Gm. per kilogram following a mismatched transfusion, and this concentration was not exceeded in the present study. It is noteworthy, therefore, that of the three hemoglobin pigments investigated, significant numbers of pigment casts were encountered in the distal convoluted tubules only in rabbits with aciduria given intravenous methemoglobin. Unlike the observation in previous experiments, none of the rabbits in these three groups died of fatal hemoglobinuric nephrosis.[4, 11, 21] The reduction in mortality, we believe, may be attributed to the fact that the animals in these experiments were not deprived of food and water for five days, and smaller doses of hemoglobin were employed.

SUMMARY

The influence of intravenous injection of old hemoglobin, modified hemoglobin, and methemoglobin on pigment cast formation was studied in three groups of rabbits. Hemoglobin of different ages stored up to 330 days was not toxic, nor did aging exert any appreciable influence on urine excretion in nine rabbits. Oxyhemoglobin which was modified by acidification and neutralization prior to intravenous injection was toxic. Seven of fourteen animals became extremely weak, one died, and two refused to eat. In spite of the immediate toxic effects, modified hemoglobin did not predispose to an accumulation of pigment casts. In the last group of ten animals significant numbers of pigment casts and increases in kidney weight were observed in five with aciduria following intravenous methemoglobin injection. Of the remaining five with alkalinuria there were significant increases in kidney weight without many pigment casts. Of the three pigments analyzed, methemoglobin is the most nephrotoxic. The mechanism by which methemoglobin exerts such a nephrotoxic effect is not apparent from these studies.

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EFFECT OF CERTAIN METABOLIC PROCESSES UPON THE CLOTTING MECHANISM

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SINCE the original discovery of heparin in dog liver by Howell and his student collaborator, McLean,[14, 23] and the demonstration of its uniquely powerful anticoagulant properties, the role of heparin in the normal clotting mechanism of human blood has been argumentative, and even today remains equivocal.

At present heparin is believed to be a nonhomogeneous mixture of mucoitin-polysulfuric-esters, consisting of glycuronic acid, glucosamine, sulfuric acid, and possibly acetic acid.[20, 21] It has a high molecular weight, and carries an exceptionally strong negative electric charge, apparently the strongest of all the organic compounds of the animal body. The strongest preparations consist of no less than 45 per cent sulfuric acid. In spite of marked ionic dissociation, heparin exerts but a small fraction of the osmotic pressure which this would lead one to expect. Such a phenomenon, exhibited in common with thymonucleic acid, allows the storage of heparin in the mast cells.

In the clotting mechanism, heparin is both antithrombic and antiprothrombic. Its action is dependent upon a co-factor,[13] or heparin complement, probably a component of the crude "albumin" fraction of the plasma proteins. In its first-phase actions, heparin probably exerts its major effect as a thromboplastic inhibitor.[12] It also has an effect on other blood systems.[21] One of its most important actions is the formation of stable salts with proteins (protamines, histones, and hemoglobin)[18] with consequent inactivation of its anticoagulant properties. Chargaff and Olson[9] reported the inactivation of heparin by the protamine, salmine, both in vitro and in vivo, and suggested its use in clinical medicine. Most of the heparin in plasma must be bound to protein and not exist in the free state.[18]

A determination of the physiologic role of heparin in normal blood clotting has been hindered by lack of a convenient assay method. The most satisfactory method is the isolation of heparin as the crystalline barium salt, and its identification by its specific anticoagulant activity on fresh, whole blood. Recently Jaques, Monkhouse, and Stewart[19] reported a chemical assay method, by which they were able to recover 80 to 90 per cent of heparin added to whole blood, and 100 per cent of that added to plasma. In normal human blood without added heparin, a value was found equivalent to 0.009 mg. per cent of heparin. It was suggested that certain modifications would be indicated in applying this method to the estimation of normal blood heparin. The metachromatic reaction of heparin with toluidine blue is relatively insensitive, and subject to interference by proteins.

Astrup and Darling[5] developed, in 1941, a very accurate method of determination of antithrombin. They suggested[6] that in addition to the normal antithrombin (among the more soluble albumins) present in blood, there also existed a "thrombin inhibitor" composed of heparin and a "thrombin co-inhibitor" (the co-factor), one of the less soluble albumins. They demonstrated that thrombin is inactivated much more rapidly by thrombin inhibitor than by antithrombin. Using the antithrombin assay method, Volkert[25] found that he could produce in rabbits a significant increase in antithrombin concentration by immunization and anaphylactic shock. He then was able to differentiate two independent components of the

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antithrombin content. He thought the first component, normally making up 80 per cent of the antithrombin content of the blood, might not be a real substance, but was possibly identical to the inhibiting properties of the plasma albumins as exerted by simple adsorption. The second component, normally making up 20 per cent of the total antithrombin, is variable, increasing under certain pathologic conditions, and shows striking points of resemblance to heparin plus co-factor. In human beings who had repeated small pulmonary emboli, he observed[26] a moderate increase in antithrombin corresponding to a doubling of heparin content of the blood. He concluded that there was no doubt regarding the identity of heparin and the variable antithrombin blood content.

The inactivation of heparin by protamine had been used by Jaques and Waters[15, 16] to determine the amount of heparin present at various stages of anaphylactic shock. They employed a saline solution of protamine, made to constant volume in various dilutions, and selected the tube with the shortest clotting time as indicating the end point. In a study of the hemorrhagic tendency in the x-irradiated dog, Allen and his colleagues[2, 3] utilized toluidine blue and protamine sulfate for inactivation in vitro and in vivo of a naturally occurring, endogenous anticoagulant which they regarded as responsible for the hemorrhagic state, and whose properties, so far as tested, were indistinguishable from those of heparin. They later noted[1, 4] wide variations in the tolerance of human blood for added heparin in certain pathologic states, and evolved a method of adding a standard amount of heparin to blood specimens and then "back titrating" with standard antiheparin (protamine sulfate) to a clotting end point. They did not claim the test was "necessarily a measure of heparin itself or even of heparinoid materials, for it may be increased by several factors, only some of which are known. . . . It appears to be a measure of a clotting defect similar to, though not identical with, that produced by the intravenous administration of a commercial beef heparin."[4] They acknowledged[1] that the protamine titration is affected by several factors, most of which influence fibrin formation. Among these are: heparin or heparin-like substances, prothrombin deficiency, impairment of the conversion of prothrombin to thrombin, hemophilia, and the absence of fibrinogen.

Smith and his associates,[24] studying the influence of therapeutic ACTH and cortisone on the clotting mechanism, frequently noted an initial transient rise in protamine titer within four hours of the first 20 mg. intramuscular injection of ACTH, with a subsequent return to control levels even though therapy was continued. The initial rise in protamine titration was often accompanied by a lengthening of the clotting time but not by consistent changes in one-stage prothrombin time, plasma-diluted prothrombin time, two-stage prothrombin content, or Ac globulin. These latter clotting factors were, however, sometimes affected if administration of the drug was continued. These investigators thought that their findings regarding the possible release of heparin-like substance into the blood stream following ACTH or cortisone administration were related to the demonstration by Dougherty and Dougherty[11] of changes in the mast cells of the experimental animal receiving cortisone.

In the course of the studies on clotting changes in myocardial infarction and its thrombo-embolic complications, diabetic patients were investigated and observations resulted which suggested a relationship between carbohydrate metabolism and the coagulation mechanism. This report is an exploration of these relationships, particularly as regards the occurrence of a heparin-like substance in the blood of normal persons.

METHODS

Clotting Time.—The Lee and White coagulation time was modified in the following manner: with a 20 ml. syringe containing 2 to 3 ml. of isotonic saline and attached to a No. 20-gauge needle, a clean venipuncture was accomplished, preferably without using a tourniquet, and the saline injected. After waiting approximately thirty seconds, blood was gently aspirated, and 1 ml. was placed in each of three saline-rinsed serological test tubes (13 by 100 mm.). The tubes were kept in a rack at room temperature, and after five minutes one

tube was gently tilted every minute (every thirty seconds after fifteen minutes had elapsed) until a firm clot had formed. This is an arbitrary point, and uniform results are obtained by having one person perform all tests. After the first tube was clotted, the second was observed, and in turn the third. The final clotting time was defined as the average of the times for clot formation in the last two tubes.

Protamine Titration.—A protamine stock solution was prepared in 100 ml. lots from protamine sulfate* in a concentration of 1 mg. per milliliter of 0.85 per cent saline, and stored under refrigeration. The stock solution should be aged twenty-four hours before using, and remains stable one to three weeks. It was then microburetted into silicone-treated tubes (12 by 75 mm.), in 0.01 ml. increments, with a range of 0.05 mg. to 0.14 mg., and the tubes adjusted to a constant volume of 0.5 ml. by adding 0.85 per cent saline.

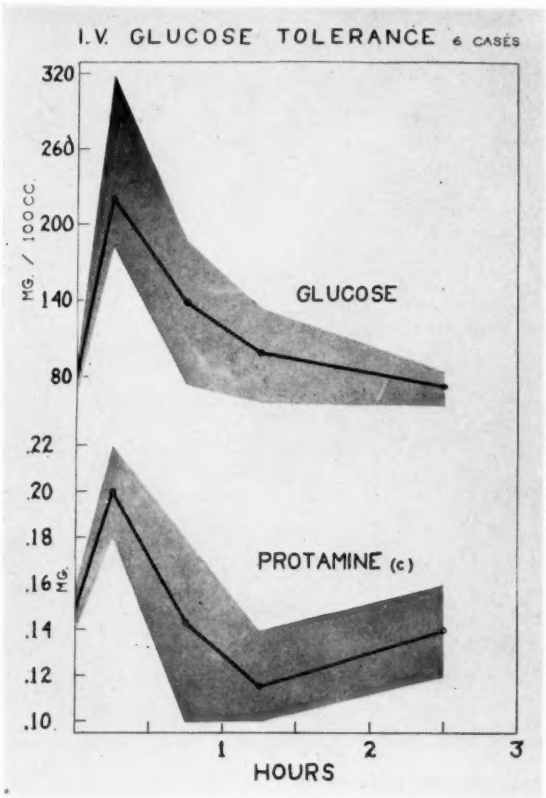

Fig. 1.—Blood glucose level and concurrent protamine titer in specimens obtained in six patients during routine glucose tolerance tests. The gray area represents the distribution of values and the black line the arithmetic means of values.

Blood was obtained as described above, except that silicone-treated syringes were used and exactly 7 ml. placed in a silicone-treated 15 ml. conical centrifuge tube, already containing 0.1 ml. (0.637 mg.) of a saline solution of heparin sodium salt (Roche). A waxed cork stopper closed the centrifuge tube, and the blood was mixed with the heparin by rolling and inverting the tube several times. Blood in this form may be used after fifteen minutes, and in the refrigerator has kept satisfactorily for as long as forty-eight hours.

By the use of a 0.5 ml. pipette, blood was added to each of the tubes of protamine solution, the tubes closed with waxed stoppers, and each inverted at least twice.

*The protamine sulfate was generously supplied by Eli Lilly & Company, Indianapolis, Ind.

The test may be read in one hour, or the reading delayed for twenty-four hours. The latter period is advantageous in that the clot separates as a distinct hanging mass. The protamine titer is defined as that tube of lowest titer in which a firm clot has formed, and expressed as protamine sulfate in milligrams per milliliter of blood. The normal for this method, which in our graphs we refer to as procedure C is 0.14 to 0.16 mg. per milliliter.

If an aqueous solution of protamine sulfate is employed, and the increments made to volume with distilled water (which we refer to as procedure A), the normal is 0.22 to 0.24 mg. per milliliter. Hemolysis is inevitable in such a system.

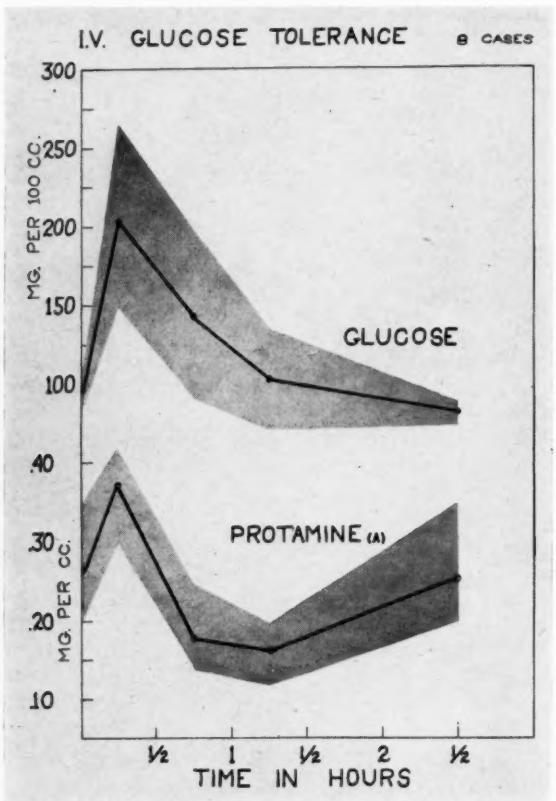

Fig. 2.—Routine glucose tolerance tests in eight patients, using an aqueous protamine system, in which hemolysis always occurs.

Miscellaneous.—(a) *Intravenous Glucose Tolerance Test:* Following the control specimen, 70 ml. of 50 per cent glucose solution were rapidly given by intravenous drip. The subsequent specimens were then obtained at 15, 45, 75, and 150 minutes, timing from the conclusion of glucose administration. Blood glucose was determined by the Folin-Wu procedure, and phosphorus by the Fiske-Subbaroff procedure, using the Evelyn photometer.

(b) *Insulin Tolerance Test:* This test was performed by the intravenous injection of 0.1 unit of regular insulin per kilogram of body weight.

(c) *Potassium:* Potassium levels were determined by the Beckman flame photometer. In this hospital's laboratory 99 per cent of normal specimens fall within 13.0 to 23.0 mg. per 100 milliliter.

RESULTS

When protamine titer measurements are made upon specimens during an intravenous glucose tolerance test (Fig. 1), the fifteen-minute maximal hyperglycemia is accompanied by a maximal rise in protamine titration. The clotting times, not graphically represented, also are lengthened at this point. The titer thereafter falls off to a point below normal, but by the conclusion of the test has risen to or above its usual level. The only test which did not produce this identical relationship was in a patient who during the glucose tolerance test received 50 mg. of cortisone parenterally. In a titration procedure in which hemolysis is inevitable, the same parallelism between glucose and titration values obtains (Fig. 2).

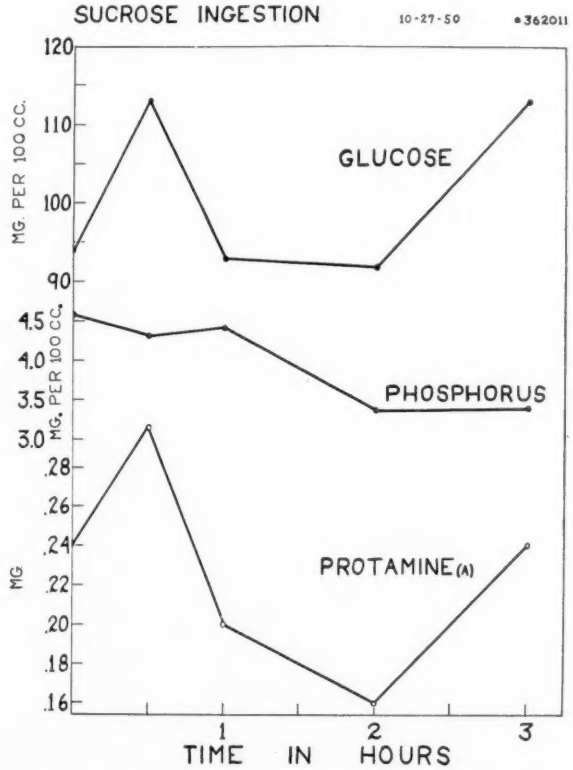

Fig. 3.—Following the ingestion of 100 Gm. of sucrose, the protamine titration curve is similar to those in Figs. 1 and 2, despite near-normal glucose values.

The blood glucose level need not approach pathologic range to produce the same pattern in the protamine titer variation. This is noted in a typical sucrose ingestion experiment (Fig. 3) in which, following 100 Gm. of sucrose, the blood glucose values remain within normal range. Similar findings result from oral glucose tolerance tests.

The apparently parallel curves of blood glucose levels and protamine titration are not reproduced if a slow intravenous drip of a dilute glucose solution

is given (Fig. 4). Under these conditions, only protamine titer depression is observed, as seen in the second phase of the glucose tolerance tests.

The insulin tolerance test (Fig. 5) produces a drop of the protamine titer below normal and a slow rise to the normal level again. These changes are possibly mediated by the hypoglycemia-induced epinephrine outflow, as demonstrated by the subcutaneous administration of epinephrine (Fig. 6). Here results a sudden drop in the protamine titer, as well as in the clotting time, and a subsequent slow rise to the normal level.

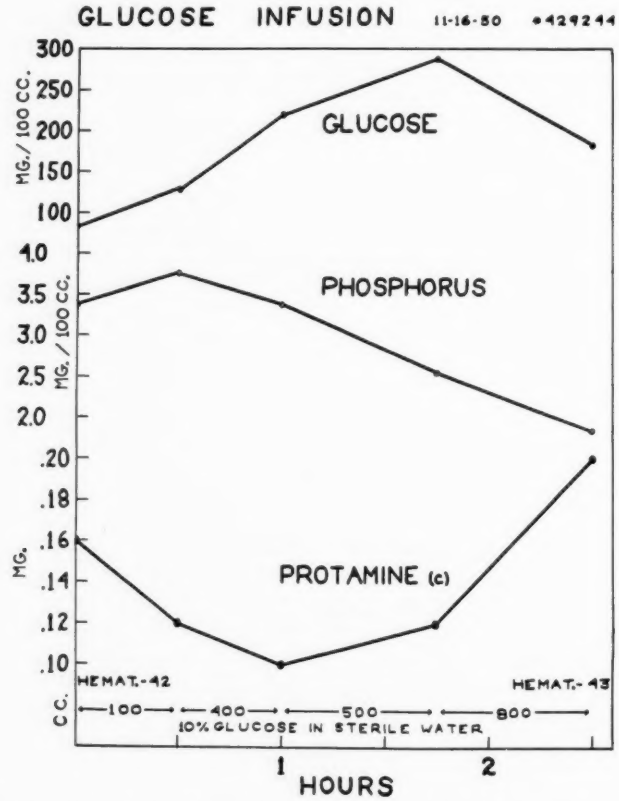

Fig. 4.—Effect of 1800 ml. of 10 per cent glucose solution intravenously. Note the slight change in hematocrit.

The effect of a single intramuscular injection of ACTH produces changes similar to those observed by Smith and associates, namely, a transient rise of protamine titration and of clotting time.

We have compared the protamine titration procedure of Allen with the procedure here in parallel determinations during a glucose tolerance test and find similar changes, but the variations by the original Allen technique do not appear as great as those demonstrated here.

The studies were carried out on persons who had no blood dyscrasia, liver disease, nor recent trauma or surgery, and in whom one would not suspect any coagulation defects.

DISCUSSION

It has been proposed by Jorpes[21] that the location of the mast cells around capillaries with their resultant ability to discharge their granular contents almost directly into the blood stream would suggest that these cells and their product, heparin, might fulfill a necessary physiological function. Thus the liberation of heparin might prevent intravascular coagulation in those very tissues where rapid metabolite exchange would tend to initiate thrombosis. If this high molecular weight polysaccharide does partake in physiologic processes,

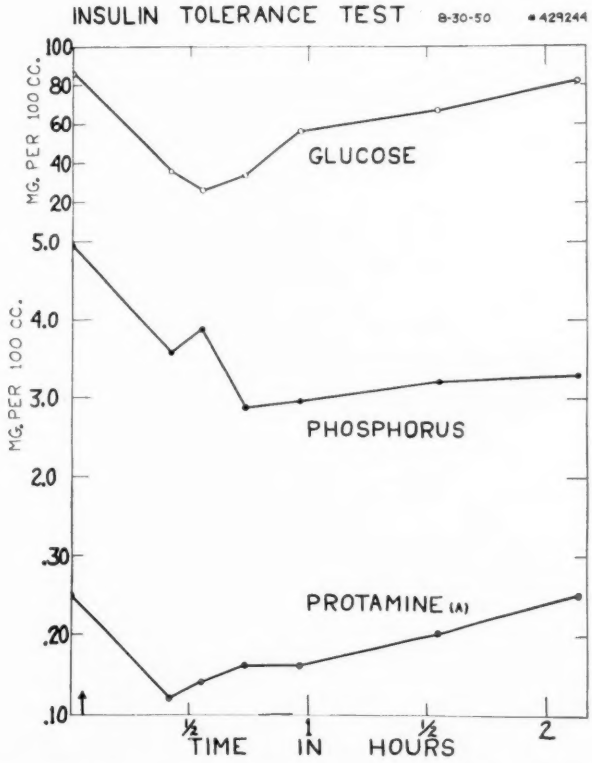

Fig. 5.—Regular insulin, 7 units, given intravenously to a normal volunteer.

its unique physiochemical properties of a high negative electric charge, its large ester sulfuric acid content, and its low osmotic pressure, would make possible multiple roles, of which a coagulation inhibitor would be only one.

The data reported here cannot be presumed to present any definitive experimental evidence for the existence of heparin in normal human blood. The protamine titration procedure does not yet rest on a firm theoretical footing, and its specificity remains to be demonstrated. Strong evidence for its validity would appear to be the prediction by its use of eventual crystalline heparin yield by Jaques and Waters in their work with the dog in anaphylactic shock.[17]

In the present work we have endeavored to modify Allen's procedure to make it more sensitive, and yet subject to less influence from minor variations

in the handling of blood. The use of siliconized glassware should decrease the ever present effect of foreign surfaces. In view of the simple mass law relationship of heparin and protamine,[18] the use of a constant, equal volume of protamine solution, as originally recommended by Jaques,[15] should markedly increase the sensitivity of the determination.

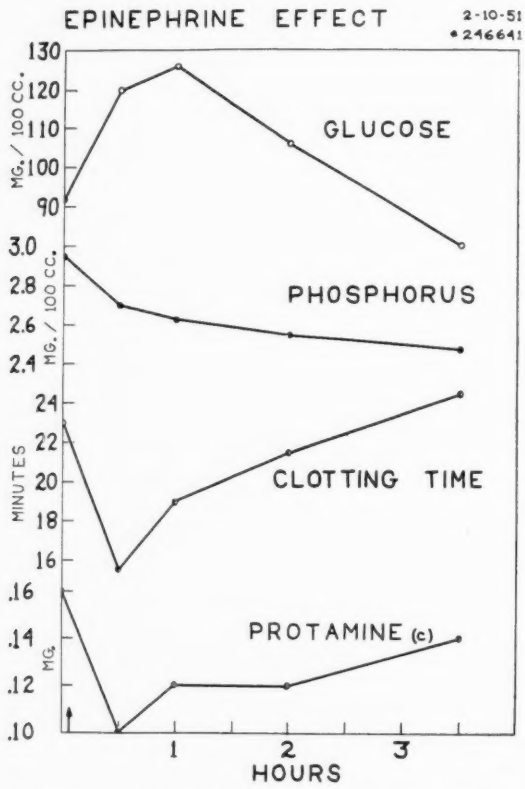

Fig. 6.—Epinephrine, 0.3 mg., given subcutaneously to a normal volunteer.

The influence on blood coagulability of hyperglycemia induced by various means has been previously reported,[7, 10, 22] but these authors ascribed the varied changes to increased blood calcium, and dilution of fibrinogen concentration by increased blood volume. The degree of change in such clotting components would be so slight as to preclude their effective influence, according to our current concepts. Cannon and Gray[8] studied the decrease in blood coagulation time induced in cats by administering epinephrine, and concluded that this agent appeared to act in some other way than by increasing blood sugar. Recent investigation[27] of this effect reported both in vitro and in vivo acceleration of coagulation by epinephrine and nor-epinephrine, but inhibition of such acceleration by the in vitro addition of adrenolytic drugs. It was, therefore, suggested that the clot-accelerating effect of these sympathomimetic compounds was a catalytic action rather than due to a change in the reacting substances themselves.

SUMMARY

A modification of the Allen protamine titration procedure is described, utilizing siliconized glassware, a smaller quantity of blood, and a constant volume of protamine solution. This modification is demonstrated to be sensitive and consistent. Its use, heretofore restricted to the investigation of hemorrhagic phenomena, permits the demonstration of constant variations during certain simple metabolic experiments.

Certain nonmorbid physiological processes, particularly involving carbohydrate metabolism, appear to exert a marked influence on the human clotting mechanism. The changes are suggestive of those involving endogenous heparin or heparin-like material in the blood, but the mechanism of production of such material cannot yet be explained.

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SEBORRHEIC DERMATITIS; A LOCAL METABOLIC DEFECT INVOLVING PYRIDOXINE

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FROM the time of its discovery, pyridoxine has been known to play an important role in the maintenance of normal mucocutaneous surfaces. In 1934, György[1] described an essential vitamin for the rat distinct from vitamins B_1 and B_2. He noted that animals fed a diet deficient in this substance developed a scaly skin and tail and swollen red paws. For this reason it was called the rat "anti-acrodynia factor." Because of the relationship of this nutrient to dermatitis, it was also designated "Adermin" until it was synthesized by Harris and Folkers.[2] It was then renamed pyridoxine. Numerous observations on deprivation studies in various animals have been reported.

Young rats on pyridoxine-deficient diets develop the following abnormalities: (a) a dermatitis which resembles human seborrheic dermatitis; (b) muscular weakness[3]; (c) a decrease in lymphoid tissue[4]; (d) a decrease in circulating antibodies[5]; and (e) an increased output of xanthurenic acid in the urine after the ingestion of tryptophane or a high protein diet.[6] Puppies develop a hypochromic anemia which responds specifically to pyridoxine[7]; and swine also develop anemia and have signs of a central nervous system disorder.[8] Monkeys exhibit a seborrhea-like dermatitis, weakness, lymphocytopenia, ploymorphonuclear leukocytosis,[9] and, like other animals,[10] excrete xanthurenic acid in the urine after the ingestion of tryptophane.[11]

During this same period, the patho-biochemical changes induced by pyridoxine deficiency have been investigated. Pyridoxine has been shown to serve both as a co-decarboxylase[12] and a transaminase,[13] and is thought to catalyze one of the steps in the transformation of tryptophane to niacin.[14]

Pyridoxine is also related to fat metabolism. The work of Burr and Burr[15] showed that rats fed a diet devoid of fat develop a scaly condition of the skin and tail, and red swollen paws which cannot be differentiated from the acrodynia described by György in vitamin B_6 deficient rats. Birch and György's report[16] suggested that a relationship exists between the two deficiencies in that certain fats cure the dermatitis produced by pyridoxine deficiency. Birch[17] examined the relationship of pyridoxine and the fatty acids of corn oil in curing rat acrodynia, and concluded that at least two factors were involved, pyridoxine, and also corn oil unsaturated fatty acids. This opinion was based on the inability of pyridoxine to cure acrodynia unless corn oil fatty acids were present in the diet. Salmon[18] indicated that the acrodynia-like dermatitis produced by a fatty acid deficiency will not clear with subsequent fatty acid ingestion if a vitamin B_6 deficiency is superimposed. Quackenbush and Steenbock[19] showed that linoleic acid from corn oil cures rat acrodynia when fed alone, and hence no pyridoxine is needed. In a later paper, Schneider, Steenbock, and Platz[20] decided that fatty acid deficiency acrodynia can be cured completely by fatty acids or partially or temporarily by pyridoxine and postulated an accessory factor to aid vitamin B_6 in complete clearing of rat acrodynia.

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The similarity of the cutaneous lesions in rats to such human dermatoses as seborrheic dermatitis and eczema; and the rather vague relationship of acne to fat metabolism, prompted numerous therapeutic trials in human cutaneous disorders. Pehl[21] used pyridoxine in juvenile seborrheic dermatitis with little success. Jolliffe and associates[22] utilized 50 to 250 mg. of pyridoxine per day in patients with acne, and reported success in a majority of patients. Wright, Samitz, and Brown[23] treated seborrhea and various dermatoses with pyridoxine with moderate success. György[24] reviewed the seborrhea-like dermatoses in man, and indicated their similarity to cutaneous lesions in animals deficient in pyridoxine, biotin, pantothenic acid, or riboflavin. He also indicated the relationship of these factors to fat and protein metabolism. Despite the use of pyridoxine in these and other therapeutic trials in human cutaneous disorders, pyridoxine deprivation studies in human beings failed to produce significant dermatitis[25]; and no clear-cut evidence was obtained regarding the importance of pyridoxine in skin metabolism. The advent of vitamin analogues led to the recognition by Ott[26] of the antipyridoxine activity in chicks of one of these antimetabolites, desoxypyridoxine. This analogue was subsequently used in human beings by Gelhorn and Jones[27] in an attempt to induce remissions in terminal cases of lymphoma. They did not observe any signs of pyridoxine deficiency. Mueller and Vilter[28] reported the effects of a vitamin B complex poor diet plus 60 to 150 mg. of desoxypyridoxine in human beings, and described the clinical syndrome of human acute pyridoxine deficiency.

The majority of desoxypyridoxine-treated patients develop an oily, scaling, erythematous seborrhea-like lesion of the scalp, brow, nasolabial folds, retroaural regions, and occasionally of the perineum. Most of the patients excrete xanthurenic acid in the urine in large amounts following a test dose of tryptophane. Absolute lymphocytopenia and a polymorphonuclear leukocytosis are common findings. A few patients develop glossitis and stomatitis which cannot be differentiated from those of niacin or riboflavin deficiency. All of these abnormalities disappear rapidly when pyridoxine is added to the experimental regime, but are unaffected by the administration of the other members of the vitamin B complex. The dermatitis is reminiscent of the skin lesions seen in pyridoxine-deficient rats.

Several unpublished observations[29] in a small number of patients will be recorded to explain the basis for setting up the experiments which will be reported in this paper. Two patients who had signs of pyridoxine deficiency induced by a diet low in vitamins of the B complex and 100 mg. of desoxypyridoxine per day developed severe seborrheic-like dermatitis symmetrically located over the brow, nasolabial folds, and retroaural areas. They also demonstrated the chemical and hematologic signs of pyridoxine deficiency mentioned above. While the diet and desoxypyridoxine were continued, pyridoxine, in water-soluble ointment base, vanibase, was applied to a small area of one side of the face in a concentration of 10 mg. per gram of base four times a day. The plain ointment base was applied to symmetrically located areas. The areas treated with pyridoxine ointment but not the control areas cleared completely in forty-eight hours. If a large area, for instance one side of the face, was treated with the pyridoxine ointment, all lesions, both local and distant, cleared rapidly.

A patient with mild spontaneous seborrheic lesions over the scalp, brow, and bearded areas was given desoxypyridoxine in vanibase in a concentration of 100 mg. per gram of base four times a day. This ointment was applied to a unilateral lesion, and a similar symmetrically placed lesion was treated with vanibase alone. In fourteen days the area treated with desoxypyridoxine developed pronounced intensification of the lesion, and the patient developed se-

vere blepharitis. Pyridoxine was administered in daily doses of 300 mg. with no improvement of the cutaneous lesions. Pyridoxine in vanibase in concentration of 10 mg. per gram was applied to the intensified lesion. It cleared completely in ten days. These observations and the morphologic similarity of the skin lesions induced by desoxypyridoxine to seborrheic dermatitis prompted these studies to determine whether an abnormality in pyridoxine metabolism might occur in this common skin disorder.

MATERIAL AND METHODS

Twenty-three patients were selected with spontaneous seborrheic dermatitis of the sicca type in which the scalp, brow, nasolabial folds, and bearded areas were covered to varying degrees with greasy adherent scales on an erythematous base. They were patients at the Cincinnati General Hospital, The Hamilton County Chronic Disease Hospital, The Dunham Tuberculosis Sanatorium, and the Longview State Hospital for mental disease. In addition to seborrheic dermatitis, five of the patients had minimal to moderate pulmonary tuberculosis, but in every case the lesions were stabilized or were improving. One patient had postencephalitic Parkinsonism, another hemiplegia, and five others had a variety of mental disorders; eleven patients with seborrheic dermatitis were uncomplicated by the presence of other illness. All were on standard hospital diets which, though varied in content, were adequate in calories, protein, fat, and vitamins. Clinical evaluation did not reveal signs of nutritional deficiency in any. The past medical records, dietary histories, and physical examinations of all the patients were reviewed, and a consulting dermatologist corroborated the diagnosis of seborrheic dermatitis of the sicca type. All patients were continued on the same diet and medications which they had received prior to these studies.

The patients were studied in two groups. Each of the eleven patients in the first group was given 300 mg. of pyridoxine hydrochloride per day for a four-week period, and was followed at weekly intervals.

The second group of twelve patients was divided into two subgroups of six patients each. No changes were made in diets or medications. Before any additional medication was given, a twenty-four hour urine specimen was collected, after which each of the patients was given 10 Gm. of dl-tryptophane by mouth and a second twenty-four hour urine specimen was collected. These urines were analyzed for 4-pyridoxic acid by the method of Huff and Perlzweig[30] and xanthurenic acid by the method of Rosen, Huff, and Perlzweig[31] as modified by Glazer and her associates.[32] Six patients received vanibase to all of their cutaneous lesions, and five concurrently received 100 mg. of pyridoxine parenterally every four hours for a three-week period. The sixth patient was given 1 Gm. of pyridoxine dissolved in 1,000 ml. of 5 per cent glucose in water by continuous intravenous drip over a fifteen- to eighteen-hour period daily for ten days. Another determination of xanthurenic acid excretion after tryptophane administration was performed, and the 4-pyridoxic acid content of the urine was also determined at the end of this period. Seven to ten days after the injections of pyridoxine were stopped the vitamin was applied in the form of an ointment in the concentration of 10 mg. per gram of vanibase to the seborrheic lesions four times a day. Whenever possible, the pyridoxine ointment was applied to one area, and a symmetrically placed similar lesion was treated with vanibase alone. In five to twenty-one days the tests for 4-pyridoxic acid and xanthurenic acid were repeated. Photographs were obtained at the onset and conclusion of each stage of treatment. The second subgroup of six patients was treated with pyridoxine ointment following tests for 4-pyridoxic and xanthurenic acids. These tests were repeated five to twenty-one days following the onset of treatment and when treatment was discontinued. When cutaneous lesions recurred, the patients were treated with 100 mg. of pyridoxine parenterally every four hours for a three-week period. Photographs of the lesions were taken at the beginning of the experiment and at the end of each period of treatment.

RESULTS

During the oral administration of 300 mg. of pyridoxine, seborrheic lesions in two patients showed slight improvement, in five patients they were unchanged, and in four patients the cutaneous lesions were intensified.

The parenteral administration of 600 to 1,000 mg. of pyridoxine resulted in only slight improvement in the cutaneous lesions of two patients. All twelve patients treated with local application of pyridoxine ointment had complete clearing of the areas treated in five to twenty-one days, although two patients required concentrations of 50 mg. of pyridoxine per gram of vanibase to eliminate the last vestiges of the dermatitis.* Itching disappeared first, then scaling, and finally the erythema. Control areas were not improved, and acne vulgaris, acne rosacea, and other skin lesions complicating the seborrheic dermatitis usually were unaffected by this ointment. As long as the ointment was continued, usually for a period of one month, the seborrheic lesions remained quiescent. After local therapy was discontinued, the lesions returned in three patients in three to four weeks, in two in six to eight weeks, and have not re-

Fig. 1.—Patient with dermatitis induced by parenterally administered desoxypyridoxine.

*Three of thirteen additional patients with seborrheic dermatitis of the sicca type treated with oral and local pyridoxine on a routine basis have failed to respond to either form of therapy. Previously, these patients had been resistant to all forms of therapy in common use, including x-ray irradiation.

curred in two patients. The other five patients could not be followed. The recurrent lesions disappeared again with the reinstitution of local pyridoxine therapy. Other types of seborrhea, particularly the moist erythematous secondarily infected type common to the flexural regions of young people, were unaffected or made worse by this ointment.

After tryptophane administration, xanthurenic acid was excreted in the urine of the patients with natural seborrheic dermatitis in somewhat larger amounts than in the urine of similarly tested control subjects from the same institutions, but the average excretion by the subjects with seborrhea did not prove to be significantly higher than by control subjects (Tables I and II).

Both parenteral administration and local application of pyridoxine rapidly returned the high xanthurenic acid levels to normal range (Table III). The pyridoxine ointment did not influence the amount of 4-pyridoxic acid in the urine of treated subjects, but parenteral administration greatly increased 4-pyridoxic acid excretion.

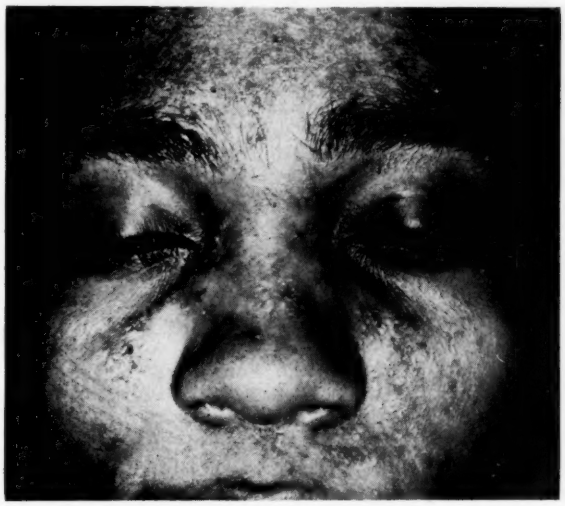

Fig. 2.—Patient with natural seborrheic dermatitis.

DISCUSSION

Rats and monkeys placed on pyridoxine-deficient diets develop scaling, erythematous, cutaneous lesions which resemble human seborrheic dermatitis. They excrete xanthurenic acid in their urine after the ingestion of tryptophane.

Human beings subjected to a vitamin B complex poor diet and a pyridoxine antagonist, desoxypyridoxine, develop a syndrome whose most constant feature is a dermatitis which cannot be differentiated from seborrheic dematitis. They also excrete xanthurenic acid in the urine in large amounts, following a test dose of tryptophane. The cutaneous lesions clear promptly with parenterally administered pyridoxine; areas treated locally with pyridoxine ointment also clear as quickly. This indicates that pyridoxine can be absorbed by the skin

and is metabolically active at the site of application. Since the coenzyme form of pyridoxine is probably pyridoxal phosphate, these observations suggest that the skin is capable of converting pyridoxine to this metabolically active form.

Natural seborrheic dermatitis does not clear with large amounts of pyridoxine administered parenterally or orally, but usually clears promptly over areas treated topically with pyridoxine ointment. These findings strongly suggest that natural seborrheic dermatitis may occur because of an increase in the local requirement for pyridoxine which cannot be met easily by oral or parenteral administration of the vitamin. So far three patients have been found who had

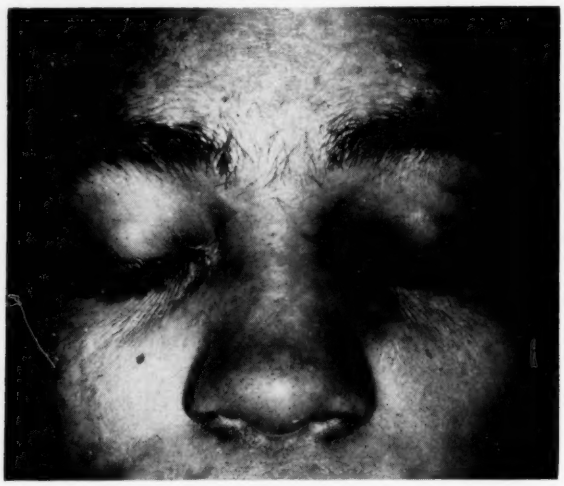

Fig. 3.—Same patient after local application of pyridoxine (10 mg. per gram vanibase) three times a day for three weeks.

TABLE I. ANALYSIS OF TOTAL TWENTY-FOUR HOUR URINARY EXCRETION OF XANTHURENIC ACID IN MILLIGRAMS AFTER THE INGESTION OF 10 GM. DL-TRYPTOPHANE

PATIENTS SERVING AS NORMAL CONTROLS					
TOTAL XANTHURENIC ACID EXCRETION IN URINE IN MG/24 HR.	F	EF	PER CENT	LOG	
206	22	21.5	97.6	2.31	
140	21	20.5	93.1	2.15	
85	20	19.5	88.5	1.93	
60	19	18.5	84.0	1.78	
34	18	17.5	79.4	1.53	
31	17	16.5	74.9	1.49	$S(x)$ 26.31
30	16	15.5	70.4	1.48	$\bar{x}$ 1.19590
27	15	14.5	65.8	1.43	$S(\bar{x}^2)$ 37.8957
17	14	13.5	61.3	1.23	$S(x-\bar{x})^2$ 6.43157
15	13	12.5	56.8	1.18	
14	12	11.5	52.2	1.15	
13	11	10.5	47.7	1.11	
11	10	9.5	43.1	1.04	
9	9	8.5	38.4	.95	
8(4)	8	6.0	27.2	.93(4)	
6	4	3.5	15.9	.78	
5	3	2.5	11.4	.70	
2	2	1.5	6.8	.30	
1	1	0.5	2.27	.00	

seborrheic dermatitis of the sicca type which failed to respond to pyridoxine ointment. These failures support the concept that multiple mechanisms may be responsible for the production of seborrheic lesions. Among these may be defective metabolism of other nutrients, disturbed cutaneous bacterial flora, and situations inducing recurrent emotional stress.

Although the average post-tryptophane excretion of xanthurenic acid by seborrheic patients did not prove to be elevated to a statistically significant degree above normal values, in several subjects high levels were obtained repeatedly. These high levels of xanthurenic acid were reduced to normal by

Fig. 4.—Same patient two weeks after discontinuation of pyridoxine ointment when dermatitis recurred.

TABLE II. ANALYSIS OF TOTAL TWENTY-FOUR HOUR URINARY EXCRETION OF XANTHURENIC ACID IN MILLIGRAMS AFTER THE INGESTION OF 10 GM. DL-TRYPTOPHANE

PATIENTS WITH SEBORRHEIC DERMATITIS					
TOTAL XANTHURENIC ACID EXCRETION IN URINE IN MG/24 HR.	F	EF	PER CENT	LOG	
268	20	19.5	97.5	2.43	
95	19	18.5	92.5	1.98	$S(x)$ 25.10
81	18	17.5	87.5	1.91	$\bar{x}$ 1.255
75	17	16.5	82.5	1.88	$S(\bar{x}^2)$ 39.9718
70	16	15.5	77.5	1.85	$S(x-\bar{x})^2$ 8.4713
64	15	14.5	72.5	1.81	
42	14	13.5	67.5	1.62	
36	13	12.5	62.5	1.56	$s^2 \frac{14.9029}{40} = .37257$
23	12	11.5	57.5	1.52	
21	11	10.5	52.5	1.32	$s = \pm .61$
17	10	9.5	47.5	1.23	
16	9	8.5	42.5	1.20	
14	8	7.5	37.5	1.15	$t = \frac{\bar{x}-\bar{x}^1}{s} \frac{nn^1}{n+n^1}$
10	7	6.5	32.5	1.00	
8	6	5.5	27.5	.90	$= \frac{.059}{.61} .3.24 + .3$
6	5	4.5	22.5	.78	
3 (2)	4	3.0	15.0	.48(2)	
1 (2)	2	1.0	5.0	.00(2)	$P = .8-.7$

TABLE III. THE TWENTY-FOUR HOUR URINARY EXCRETION OF XANTHURENIC ACID AFTER TRYPTOPHANE ADMINISTRATION TO PATIENTS, BEFORE TREATMENT AND AFTER BOTH LOCAL AND PARENTERAL ADMINISTRATION OF PYRIDOXINE

SIX PATIENTS WITH SEBORRHEIC DERMATITIS WITH ELEVATED XANTHURENIC ACID EXCRETION		
BEFORE TREATMENT XANTHURENIC ACID EXCRETION (MG./24 HR.)	AFTER 3 WEEKS OF LOCAL APPLICATION OF PYRIDOXINE OINTMENT XANTHURENIC ACID EXCRETION (MG./24 HR.)	AFTER 3 WEEKS ADMINISTRATION OF 600 MG. PYRIDOXINE PER DAY PARENTERALLY XANTHURENIC ACID EXCRETION (MG/24 HR.)
75	3	2
268	2	–
95	11	–
81	31	6
70	14	2
64	-	8

either parenteral or local administration of pyridoxine. Therefore, one must assume that pyridoxine reaches the blood stream in significant concentration from the skin. Although the parenteral administration of pyridoxine returns xanthurenic acid excretion to normal, it has little or no effect upon the seborrheic lesions. This would seem to indicate an inability to attain sufficient concentration of pyridoxine in the skin unless it is applied locally. Thus the difficulty in pyridoxine metabolism in patients with natural seborrheic dermatitis may be one of, or a combination of two or three of the following possibilities: (1) an increased requirement for pyridoxine in the skin perhaps because of a local disorder in fat metabolism; (2) an increase in the destruction of pyridoxine in the skin; (3) an increase in the "barrier" to pyridoxine between blood stream and skin.

The relationship of fat metabolism and in particular of unsaturated fatty acids to human seborrheic dermatitis also remains unexplained. Identical lesions can be produced in rats by either fat or pyridoxine deficiency, and neither of these factors singly can cure the cutaneous lesion when the animals are deficient in both. These observations indicate a close relationship between fat metabolism and pyridoxine. Whether this pertains to human cutaneous lesions remains open for future investigation.

CONCLUSIONS

These studies indicate that pyridoxine is metabolically active, or can be converted to a metabolically active substance possibly pyridoxal phosphate, in the skin. One usually considers the liver as the principal site for such conversions and rarely considers the skin in such a capacity.

These studies strongly suggest that a large percentage of patients with seborrheic dermatitis of the sicca type have a metabolic defect in the skin, possibly involving fat metabolism which increases the local requirement for pyridoxine. For unknown reasons this requirement cannot be met easily by oral or parenteral administration of the vitamin, but local application is exceedingly effective.

Acne rosacea, acne vulgaris, psoriasis, and the moist infected flexural seborrheic dermatitis of young people are not usually benefited by pyridoxine

ointment of the type used in these studies. Moist, weeping lesions should not be treated with this ointment.

In a broader sense, these studies present a concept of disease due to a local deficiency state possibly occurring because of an inborn or acquired metabolic error, which greatly increases the requirement for an essential nutrient.

We wish to thank Dr. Augustus Gibson and Dr. R. A. Peterman of Merck and Company, Inc., for the pyridoxine and desoxypyridoxine used in these studies.

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THE MECHANISM OF ACTION OF DESOXYEPHEDRINE ON THE VASCULAR BED IN THE LIMB OF THE DOG*

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THE effects of intra-arterial administration of desoxyephedrine (N-methyl-β-phenylisopropylamine) upon the rate of femoral arterial blood flow of dogs were observed and compared with those of epinephrine and nor-epinephrine under various conditions. This study was undertaken in an attempt to clarify the mechanism of the peripheral action of desoxyephedrine and to find an explanation for the phenomenon of tachyphylaxis and the reversal of the pressor action.

METHODS

Forty mongrel dogs weighing between 8 and 22 kg. were anesthetized intravenously with 35 mg. per kilogram of sodium pentobarbital. The blood pressure was recorded from the brachial artery with a mercury manometer. The mean rate of blood flow through the femoral artery was measured or recorded with a rotameter type of flowmeter† inserted between the cut ends of the divided vessel. Five milligrams of heparin per kilogram were administered intravenously every hour during the experiments.

Fresh solutions of desoxyephedrine, epinephrine, and nor-epinephrine‡ were prepared for each experiment from the crystalline compound in the *l* form as the hydrochloride or bitartrate salt. The concentrations of the solutions were usually adjusted so that the respective doses of 0.05 μg of either epinephrine or nor-epinephrine per kilogram or 10 μg of desoxyephedrine per kilogram were dissolved in 0.5 ml. of 0.9 per cent sodium chloride solution.

In a typical experiment, the flow rate through a femoral artery was recorded when the animal was in the resting anesthetized state. One-half milliliter of epinephrine solution was injected in five seconds into the tube leading to the distal portion of the femoral artery, and the changes in flow rate recorded. After it returned to the control level, a similar injection of epinephrine or nor-epinephrine was given. Then two or three doses of desoxyephedrine were injected, and finally the epinephrine and nor-epinephrine doses were repeated.

Sympathetic denervation of the vascular bed under consideration was achieved surgically by ipsilateral excision of the sympathetic chain from the twelfth thoracic to the last sacral ganglia. The effectiveness of this procedure was demonstrated by observing the increased resting rate of blood flow through the denervated limb.

Adrenergic blockade was accomplished by the intravenous administration of 0.8 to 2.0 mg. per kilogram of dihydroergotamine (D.H.E. 45) methanesulfonate. The criterion for complete adrenergic blockade was the lack of flow rate response to the intra-arterial injection of nor-epinephrine.

RESULTS

The mean arterial pressure remained stable following the intra-arterial injections of these compounds because of the small amounts employed. The

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†Fischer and Porter Co., Hatboro, Pa.

‡The compounds used in this study were supplied as follows: *l*-epinephrine, *l*-nor-epinephrine, and *l*-desoxyephedrine by Winthrop-Stearns, Inc., New York, N. Y.; Dihydroergotamine by Sandoz Pharmaceuticals, New York, N. Y.; Heparin by Abbott Laboratories, North Chicago, Ill.

effects of 300 injections of epinephrine, nor-epinephrine, and desoxyephedrine were recorded and measured. The control flow rates in the femoral artery were usually between 30 and 70 ml. per minute, but as much as 150 ml. per minute has been recorded. Because of the wide range of the control values, the results are expressed as the per cent change from the control flow rates. When the control values were less than 20 ml. per minute, the results were not considered reliable because of the large error of calibration in this range. They were not included in the analysis of the data.

The transient depression of flow rates during injections of these amines and of 0.9 per cent sodium chloride solution was considered to be an artefact from the back pressure exerted on the flowmeter float by these injections.

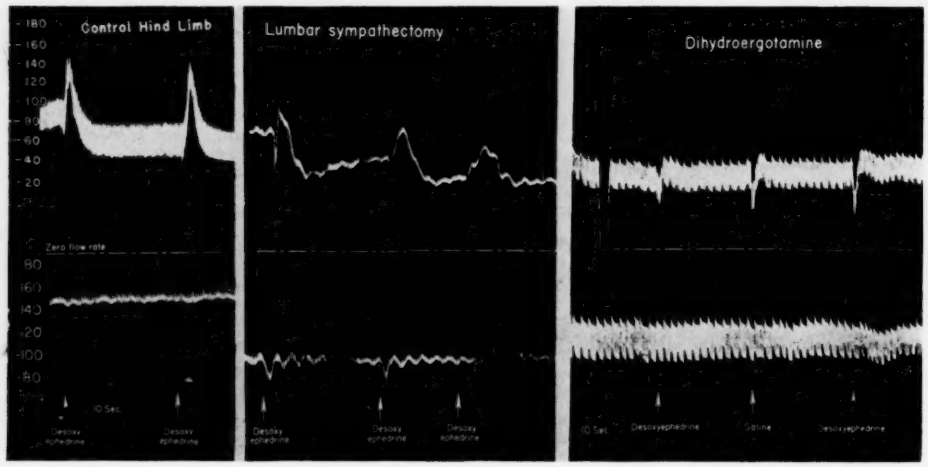

Fig. 1.—The effects of repeated intra-arterial doses of desoxyephedrine upon the femoral arterial flow rate of anesthetized dogs. *A*, Untreated resting limb; *B*, sympathectomized limb; *C*, limb treaed with D.H.E. 45.

The effects of desoxyephedrine on the flow rates are shown in Table I and Fig. 1. The typical biphasic response in an untreated resting limb consisted of a primary increase which lasted approximately one minute followed by a secondary decrease which persisted for at least five minutes. Second doses administered during this secondary decrease usually produced a greater increase followed by little, if any, decrease (Fig. 1, *A*). If the decrease following the second dose exceeded 30 per cent, a third dose was then administered which elicited invariably only a transient increase.

In the sympathectomized limb the initial dose of desoxyephedrine induced little, if any, primary increase, but caused a profound and sustained secondary decrease in flow rate. Subsequent doses usually yielded responses similar to those in untreated resting limbs (Fig. 1, *B*). In the preparations treated with D.H.E. 45, initial and subsequent doses of desoxyephedrine caused no significant change in flow rate (Fig. 1, *C*).

Epinephrine and nor-epinephrine caused a marked decrease in flow rate which usually lasted less than one minute in both the untreated resting and the

sympathectomized limbs (Table II and Fig. 2, *A*). This type of response was unaltered when these amines were given during the phase of decreased flow rate following desoxyephedrine. The per cent decreases in flow rates following epinephrine and nor-epinephrine were larger than that of desoxyephedrine. A secondary increase was often observed following epinephrine but was rarely seen with nor-epinephrine. In D.H.E. 45 treated preparations, epinephrine yielded a transient increase and nor-epinephrine elicited no response (Fig. 2, *B*). With incomplete adrenergic blockade, nor-epinephrine produced a slight decrease of flow rate. In such preparations desoxyephedrine caused a corresponding increase of flow rate (cf. Tables I and II).

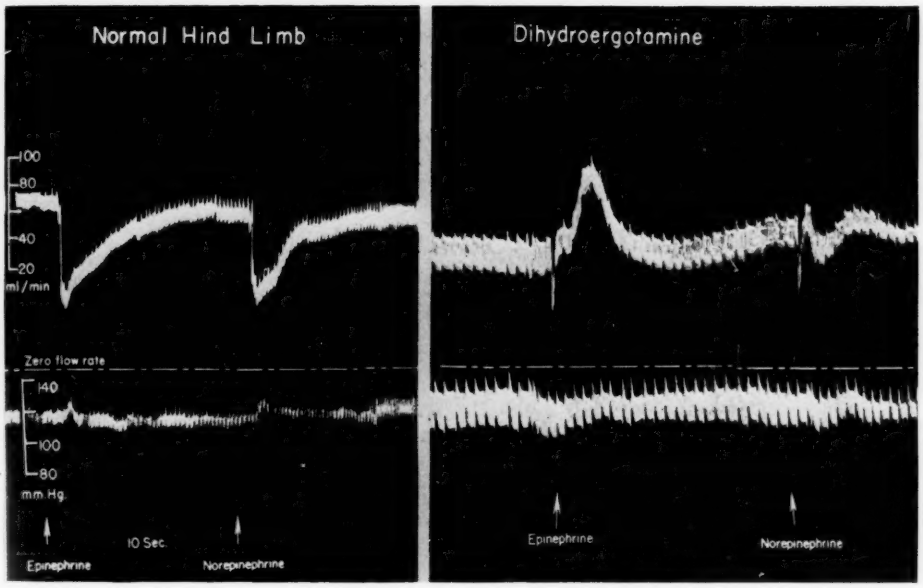

Fig. 2.—The effects of intra-arterial doses of epinephrine and nor-epinephrine upon the femoral flow rate of anesthetized dogs. *A*, Untreated resting limb; *B*, limb treated with D.H.E. 45.

TABLE I. EFFECT OF DESOXYEPHEDRINE ON RATE OF FEMORAL ARTERIAL BLOOD FLOW

	INITIAL DOSE					SUBSEQUENT DOSE				
	NO. EXP.	PER CENT INCREASE		PER CENT DECREASE		NO. EXP.	PER CENT INCREASE		PER CENT DECREASE	
		AV.	RANGE	AV.	RANGE		AV.	RANGE	AV.	RANGE
Control	10	130	38-280	46	28-72	9	230	165-400	4	0- 7
Sympathectomy	8	14	0- 53	67	55-87	5	115	48-280	17	0-52
D.H.E. 45	5	19	0- 42	4	0- 9					

DISCUSSION

Local changes in femoral arterial flow rate produced by intra-arterial injections of these amines could be analyzed more precisely than the changes in arterial pressure elicited by intravenous administration because the latter represent the combined cardiac, peripheral and central nervous system actions.

TABLE II. EFFECTS OF EPINEPHRINE AND NOR EPINEPHRINE ON RATE OF FEMORAL ARTERIAL BLOOD FLOW

	EPINEPHRINE									NOR-EPINEPHRINE					
	BEFORE DESOXYEPHEDRINE					AFTER DESOXYEPHEDRINE				BEFORE DESOXYEPHEDRINE			AFTER DESOXYEPHEDRINE		
	NO. EXP.	PER CENT DECREASE		PER CENT INCREASE		NO. EXP.	PER CENT DECREASE		PER CENT INCREASE		NO. EXP.	PER CENT DECREASE		NO. EXP.	PER CENT DECREASE	
		AV.	RANGE	AV.	RANGE		AV.	RANGE	AV.	RANGE		AV.	RANGE		AV.	RANGE
Control	16	66	50-85	22	0-120	7	56	24-69	67	0-155	20	72	40-100	6	69	38-85
Sympathectomy	4	75	55-87	7	0- 30	1	56		52		4	81	73- 95	3	72	59-90
D.H.E. 45	5	0	0	95	18-150						5	25	17- 38			

Since the systemic arterial pressure remained unchanged, it was assumed that an increased flow rate was the result of decreased peripheral resistance, i.e., vasodilatation. Conversely, a decreased flow rate could be considered as the result of vasoconstriction.

Judging from the results in the untreated resting and the sympathectomized limbs, the mode of action of desoxyephedrine apparently differs from that of epinephrine and nor-epinephrine. Successive doses of desoxyephedrine cause an increasing transient dilatation but the ability of such doses to elicit further constriction diminishes and finally disappears. These effects are in sharp contrast with the transient constriction seen regularly after epinephrine and nor-epinephrine.

The hypothesis of Gaddum and Kwiatkowski[1] concerning the mode of action of ephedrine could be applied satisfactorily to desoxyephedrine. Constriction could result from competition by this compound for the enzyme responsible for the destruction of the sympathetic mediator (possibly amine oxidase), thereby protecting the sympathetic mediator. Desoxyephedrine could cause dilatation by antagonizing the action of the mediator by "combining with the motor receptors and blocking them up." This latter combination apparently does not activate the receptors.

SUMMARY

The effects of the intra-arterial injection of desoxyephedrine and other amines upon the rate of femoral arterial blood flow were studied in anesthetized dogs.

The mechanism of action of desoxyephedrine appears to be the competitive inhibition of the sympathetic mediator at two loci: (a) the sympathetic receptors, and (b) the route of destruction or elimination of the mediator.

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THE ACUTE CARDIOVASCULAR DYNAMICS OF A FISTULA BETWEEN THE PULMONARY ARTERY AND THE LEFT AURICLE

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IN A number of congenital diseases of the heart, blood is shunted from the right side of the heart to the systemic circulation without passing through the lungs. The presence of this unoxygenated blood in the systemic vessels results in an increased cardiac output, and these together may place an abnormal stress on the circulation. Adequate understanding of these disorders has been difficult to achieve, since congenital anomalies of the heart are usually not simple malformations even at birth. By the time they present themselves for study, such patients often have extremely complicated alterations in cardiovascular dynamics. These result from the abnormal pressure relationships and changes in the cardiac output, producing myocardial hypertrophy and other modifications. Extravascular adjustments such as polycythemia and hypervolemia also contribute to the difficulty of analyzing the dynamics of these disorders.

In order to study the effects of these simple stresses on the circulation in a relatively uncomplicated fashion, we have assayed the acute effects of several relatively simple cardiovascular shunts.[1, 2] In the present report we present data on the dynamic effects of a shunt between the pulmonary artery and the left auricle.

METHOD

Nine dogs weighing 15 to 20 kg. were anesthetized with intravenous sodium pentobarbital (25 mg. per kilogram). Artificial respiration via a tracheal cannula was begun after opening the chest in the third or fourth left interspace. The ribs were retracted and the pericardium was opened over the pulmonary artery. Heparin† (5 mg. per kilogram) was given intravenously to prevent clotting. The left pulmonary artery was isolated, ligated, and a cannula introduced just distal to its origin. This cannula was attached to the inflow tube of a rotameter[3]; the outflow tube was attached to a cannula which was inserted into the left auricular appendage by means of a purse-string suture. In this manner a shunt was made between the left pulmonary artery and the left auricle (PA-LA shunt); the volume of shunt flow being measured by the rotameter. The shunt was opened from time to time, and simultaneous measurements were made of blood pressures and flow.

All pressures were recorded with Hamilton manometers[4] on a halide paper recording kymograph. Right auricular pressure was obtained via a woven nylon catheter introduced

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TABLE I. THE DYNAMIC EFFECTS OF THE OPENING AND CLOSING OF AN ACUTE SHUNT BETWEEN THE PULMONARY ARTERY AND LEFT AURICLE IN THE DOG

	TIME OF READING AFTER SHUNT OPENED & CLOSED (MIN.)	DURATION OF OPEN SHUNT (MIN.)	RANGE OF SHUNT FLOW (ML./MIN.)	SHUNT FLOW AT TIME OF MEASUREMENT (ML./MIN.)	AORTIC PRESSURE (MM. HG.)	PULMONARY ARTERY PRESSURE (MM. HG.)	LEFT AURICULAR PRESSURE (MM. HG.)	RIGHT AURICULAR PRESSURE (MM. HG.)	HEART RATE (BEATS/MIN.)
1/12/50									
Control	–	–	–	0	110/80	16/8	1/–1	1/–1	160
Shunt open	4	7	180-800	800	110/80	16/8	1/–1	1/–1	150
Shunt closed	1	–	–	0	110/80	16/8	1/–1	1/–1	150
2/23/50									
Control	–	–	–	0	115/80	25/10	3/0	2/–1	160
Shunt open	5	5	200-750	750	105/75	25/8	3/0	2/0	140
Shunt closed	½	–	–	0	105/75	32/10	3/0	2/0	150
3/27/50*									
Control	–	–	–	0	130/115	24/8	3/0	1/–1	150
Shunt open	2	6	150-400	400	125/110	22/6	4/1	1/–1	140
Shunt closed	1	–	–	0	115/80	18/8	2/0	1/–1	125

*O_2 content of aorta during control = 10.9 vol. per cent (capacity 11.9 vol. per cent); O_2 content of pulmonary artery = 4.7 vol. per cent; A-V oxygen difference = 6.2 vol. per cent. When shunt was open O_2 content of aorta was 7.9 vol. per cent, of pulmonary artery 3.1 vol. per cent and A-V oxygen difference = 4.8 vol. per cent.

into the external jugular vein and passed under fluoroscopic control into the right auricle; systemic pressure via a similar catheter placed in the root of the aorta through the carotid artery; left auricular pressure via a catheter placed in the left auricle by way of a small branch of the pulmonary vein; pulmonary pressure via a needle placed through the cannula in the pulmonary artery in such a manner that the pressure in the main pulmonary artery was recorded. In a few instances oxygen samples were obtained before and during the opening of the PA-LA shunt and analyzed for oxygen content by the method of Van Slyke and Neill.[5] Electrocardiograms (ordinary limb leads) were recorded in several experiments.

RESULTS

No marked changes in the systemic arterial, the pulmonary arterial, and right and left auricular pressures occurred in any of the nine animals, even with PA-LA shunts as great as 800 c.c. per minute, and lasting as long as seven minutes. This is shown in three representative experiments in Table I.

The absence of significant change occurred despite the decrease in arterial oxygen saturation to as low as 60 per cent. Although the experiments were carried out on animals which had a modified pulmonary system inasmuch as one lung was separated from the circulation, the pulmonary arterial pressure remained within the normal ranges.

Progressive peripheral vascular collapse occurred at a more rapid rate in these shunt experiments than in others similarly prepared but without a shunt. The possible contribution of the successive bouts of hypoxemia and hypercapnia in this susceptibility to shock remains to be determined.

Only slight changes were observed in the standard limb lead electrocardiograms. These consisted of some increase in height of P_2 and P_3, an increase in amplitude of T_2 and T_3, and elevation of S-T in these leads. These changes were restored to normal on closing the shunt.

An increase in the intrapulmonary pressure produced by constriction of the air outlet from the tracheal cannula resulted in an immediate and marked increase in flow through the shunt.

DISCUSSION

The present results demonstrate that a fairly large shunt between the pulmonary artery and the left auricle have no immediate notable effects on the heart or circulation. This result is probably due in part to the fact that the resistance through the pulmonary circuit is normally so low that the provision of a shunt whereby pulmonary artery blood enters the auricle under a slightly higher pressure has no significant effect on the pulmonary arterial pressure. The relative unsaturation of the systemic blood which becomes apparent on opening the shunt appears to have little immediate effect on the circulation in the body or on the heart itself under ordinary circumstances. The undoubted reduction of oxygen saturation of the blood entering the coronary circuit gave no immediate evidence of significant deleterious effects on the heart itself. However, this effect may have contributed to the progressive development of the shock which was noted.

The fact that the opening of the flowmeter circuit produced no fall in pulmonary blood pressure re-emphasizes the fact that the pulmonary circuit provides an extremely low resistance to flow. The lack of change in opening or closing the shunt further calls attention to the fact that there is no notable major change in the resistance of the lung circuit when the shunt is opened as compared to that existing when the shunt is closed and blood goes through the right lung only.

It is likely, however, that the opening of a pulmonary artery-left auricular shunt with its consequent reduction in oxygen saturation of the systemic blood may result in a marked redistribution of the systemic flow pattern, as was seen in our experiments on the effect of hypoxia in the dog.[6]

In conditions such as atelectasis, or in lobar pneumonia, unoxygenated blood may pass through the unaerated lung to the systemic circulation without apparent effect upon the cardiodynamics as a whole. However, the resistance to flow through such abnormal pulmonary parenchyma is probably increased, effectively limiting the flow through this part.

The relatively rare clinical condition of pulmonary arteriovenous fistula has recently been reviewed by Giampolo.[7] This syndrome is seen especially in young men who show cyanosis, especially on effort, clubbing of the fingers, polycythemia, hypervolemia, dyspnea, hemoptysis, epistaxis and, on occasion, syncope. Some of these changes have been produced experimentally by Mendlowitz.[8] These cases resemble the pulmonary artery-left auricular shunt produced in our experiments in that there is no evidence of significantly changed venous or arterial pressures.

The experiments in which increased intrapulmonic pressure resulted in an increase in the shunt flow suggest one mechanism for the syncope which is seen clinically in such types of cases. Thus increased intrapulmonic pressure, which might occur in straining or coughing, would result in an increased flow of blood through the shunt, with marked reduction in flow through the lungs leading thereby to brusk and marked acute hypoxemia.

SUMMARY

A method for the production of acute cyanosis by means of an anastomosis between the pulmonary artery and the left auricle is described.

Despite the presence of the abnormal shunt and the reduction in the amount of oxygen in the arterial blood, no marked changes occur acutely in the pressures in the right and left auricles and in the pulmonary artery and the aorta. However, an increased tendency to the development of shock was noted.

Some similarities between the artificial shunt and the clinical conditions in which deoxygenated blood bypasses the lungs, as in pulmonary arteriovenous anastomosis, are discussed.

A mechanism for the production of syncope in such types of shunt is suggested.

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LABORATORY METHODS

MICROMETHOD FOR DETERMINATION OF DENSITY OF BODY FLUIDS OF LOW DENSITY

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IN 1938,[1] the author reported a method for determining the density of serum and plasma by a simple falling drop method. This method was unique in that an oil was used which has a boiling point of over 220° C. and which is stable. This permitted a single standardization, and eliminated the constant use of nomograms and of standard solutions. In 1941,[2] this technique was adapted for study of the density of whole blood.

In the present study, the method was modified for the study of body fluids of low density, such as spinal fluid, transudates, some exudates, urine, ascitic fluid, etc. The procedure takes only a few minutes and gives results which are more accurate than ordinarily needed for such fluids. Because only very small quantities of fluid are required (0.03 ml. suffices for a single determination), the method is ideally suited for use when small quantities are available, and especially in pediatrics.

The method is based upon Stokes's law. The latter states that the rate of fall of a small solid sphere in a viscous medium is a function of the radius and the specific gravity or density of the sphere, the specific gravity and viscosity of the medium, and the acceleration due to gravity. The method described below provides that a drop of fluid be timed as it falls freely through an oil with which it is not miscible. A calibrated pipette in a special pipette holder is used to deliver a drop of constant volume and therefore of constant radius. Since the density and viscosity of the medium are constant, the density of the drop may then be determined from its rate of fall. Temperature is an important factor. Since the density and viscosity of the medium vary in a constant manner with the temperature, and since the density of the falling drop varies in a relatively constant manner with the temperature, an over-all correction can be made for any temperature at which the test is done within the range given below.

MATERIALS AND METHOD

A mixture of methyl salicylate and high viscosity mineral oil was prepared* which had a specific gravity of 0.9965 25°/25° C. The apparatus used is shown in Fig. 1. The central test tube is held firmly in a water jacket to provide for more uniform temperature. This tube must have a uniform inside diameter of 14 mm. and be 16 cm. long. It is etched with two rings exactly 10 cm. apart, the lower one being 15 mm. above the bottom of the tube. The water jacket should have a stirring rod and a thermometer accurate to 0.1° C. between

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*Prepared according to our specifications by the E. H. Sargent & Company, Chicago, Ill.

20° C. and 30° C. The central test tube is filled with the oil to approximately 15 mm. from the top of the tube. The temperature of the specimens of fluid to be tested should be allowed to come to equilibrium with the central test tube by suspending them into the water jacket.

The pipette holder (Fig. 1) is most conveniently made in the shape of a gun and equipped with rubber tubing one end of which is plugged. By turning the screw in the pipette holder the air in the tubing is displaced and a good suction apparatus is thereby made available. The pipette fits into the open end of the tubing. The pipette itself is made from unconstricted capillary tubing calibrated to deliver exactly 0.015 ml. between two etched rings on the pipette.

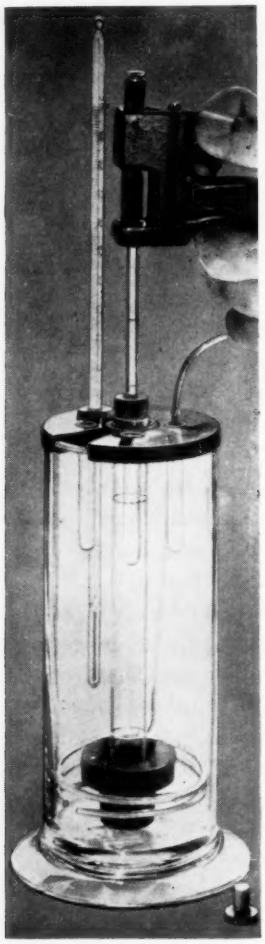

Fig. 1.—Apparatus used for determination of density of body fluids.

Standardization.—In standardizing the oil or in testing various specimens the procedure is the same. The test material is drawn into the pipette to the level of the upper ring. The tip of the pipette is then placed under the surface of the oil (Fig. 1). The pipette is then discharged until the fluid level is at the lower ring. This places a drop of 15 cu. mm. in the oil. This drop will separate from the pipette when the latter is withdrawn from the oil. Care should be taken to withdraw the pipette in such manner that the drop separates from it in the middle of the central tube. The drop will then fall freely through the oil.

The time required for the drop to pass between the two lines on the central test tube is determined with a stop watch. The temperature of the water jacket should be recorded at the time of the determination.

In order to standardize the oil, standard solutions of sodium chloride are made so that they cover the range from 1.0000 to 1.0230 25°/25° C. Their densities should be determined accurately to 10^{-4} by pyknometry. These solutions are timed as they fall through the oil at each degree of temperature from 20° to 30° C. The reciprocal of their falling time is plotted against the density of the solutions for each temperature. This yields a perfect linear relationship at each temperature.

TABLE I. CONSTANTS FOR DETERMINATION OF SPECIFIC GRAVITY OF FLUIDS OF LOW SPECIFIC GRAVITY FROM THE FALLING TIME

TEMP. °C.	K	d	TEMP. °C.	K	d
20.0	.398	.9997	25.0	.338	.9965
20.1	.397	.9996	25.1	.337	.9965
20.2	.396	.9995	25.2	.336	.9964
20.3	.395	.9995	25.3	.335	.9963
20.4	.394	.9994	25.4	.334	.9963
20.5	.393	.9993	25.5	.333	.9962
20.6	.391	.9993	25.6	.332	.9962
20.7	.390	.9992	25.7	.331	.9961
20.8	.389	.9991	25.8	.329	.9961
20.9	.388	.9991	25.9	.328	.9960
21.0	.387	.9990	26.0	.327	.9960
21.1	.385	.9990	26.1	.326	.9959
21.2	.384	.9989	26.2	.325	.9959
21.3	.383	.9989	26.3	.324	.9958
21.4	.382	.9988	26.4	.323	.9957
21.5	.381	.9987	26.5	.322	.9957
21.6	.380	.9987	26.6	.321	.9956
21.7	.379	.9986	26.7	.320	.9956
21.8	.378	.9986	26.8	.319	.9955
21.9	.376	.9985	26.9	.317	.9955
22.0	.375	.9984	27.0	.316	.9954
22.1	.374	.9984	27.1	.315	.9954
22.2	.373	.9983	27.2	.314	.9953
22.3	.372	.9982	27.3	.312	.9953
22.4	.371	.9981	27.4	.311	.9952
22.5	.369	.9981	27.5	.310	.9952
22.6	.368	.9980	27.6	.308	.9952
22.7	.367	.9979	27.7	.306	.9951
22.8	.366	.9979	27.8	.304	.9951
22.9	.364	.9978	27.9	.302	.9950
23.0	.363	.9977	28.0	.299	.9950
23.1	.362	.9977	28.1	.297	.9949
23.2	.360	.9976	28.2	.295	.9949
23.3	.359	.9976	28.3	.292	.9948
23.4	.358	.9975	28.4	.290	.9948
23.5	.357	.9974	28.5	.287	.9948
23.6	.355	.9974	28.6	.284	.9947
23.7	.354	.9973	28.7	.282	.9947
23.8	.353	.9973	28.8	.279	.9946
23.9	.352	.9972	28.9	.276	.9946
24.0	.351	.9971	29.0	.274	.9945
24.1	.350	.9971	29.1	.271	.9945
24.2	.348	.9970	29.2	.269	.9945
24.3	.347	.9970	29.3	.266	.9944
24.4	.346	.9969	29.4	.264	.9944
24.5	.345	.9968	29.5	.261	.9943
24.6	.344	.9968	29.6	.259	.9943
24.7	.342	.9967	29.7	.256	.9942
24.8	.341	.9966	29.8	.253	.9942
24.9	.340	.9966	29.9	.250	.9941
			30.0	.249	.9941

TABLE II. DIRECT READINGS OF SPECIFIC GRAVITY FROM FALLING TIME AND TEMPERATURE (BODY FLUIDS OF LOW SPECIFIC GRAVITY)

SP. GR.	20	21	22	23	24	25	26	27	28	29	30
1.023	17.0	16.2	15.3	14.4	13.5	12.7	12.2	11.4	10.3		
1.022	17.7	16.8	15.9	14.9	14.1	13.2	12.6	11.8	11.0	10.0	
1.021	18.6	17.6	16.6	15.6	14.7	13.8	13.1	12.3	11.5	10.4	
1.020	19.5	18.5	17.4	16.4	15.4	14.4	13.6	12.8	11.9	10.8	
1.019	20.6	19.4	18.3	17.0	16.0	15.0	14.3	13.4	12.4	11.2	10.0
1.018	21.7	20.5	19.2	18.0	16.8	15.7	14.8	14.0	13.0	11.7	10.4
1.017	23.0	21.5	20.2	18.8	17.7	16.5	15.6	14.6	13.6	12.2	10.9
1.016	24.2	22.8	21.7	19.9	18.6	17.3	16.4	15.4	14.3	12.7	11.4
1.015	25.8	24.3	22.7	21.0	19.7	18.3	17.3	16.2	15.0	13.4	11.8
1.014	27.7	26.0	24.2	22.4	20.8	19.4	18.3	17.0	15.7	14.1	12.5
1.013	29.7	27.7	25.8	23.8	22.1	20.5	19.3	18.0	16.6	14.8	13.3
1.012	32.0	30.0	27.7	25.5	23.7	21.8	20.5	19.0	17.6	15.7	13.9
1.011	34.8	32.4	29.9	27.4	25.3	23.4	21.9	20.2	18.7	16.6	14.7
1.010	38.0	35.3	32.5	29.7	27.2	25.0	23.4	21.8	20.0	17.7	15.7
1.009	42.0	38.8	35.5	32.2	29.6	27.0	25.1	23.3	21.4	18.9	16.8
1.008	47.0	43.1	39.2	35.7	32.4	29.5	27.4	25.1	23.0	20.4	18.0
1.007	56.0	48.2	44.0	39.0	35.7	32.2	29.8	27.2	25.0	21.9	19.4
1.006	62.0	55.0	49.4	43.9	39.4	35.8	32.8	29.8	27.2	23.9	21.0
1.005	72.0	64.0	57.0	49.8	44.0	40.0	36.3	32.8	30.0	26.0	23.0
1.004	87.0	77.0	66.0	58.0	51.0	45.2	41.0	36.8	33.2	28.8	25.4
1.003	110.0	96.0	80.0	68.0	59.0	52.0	46.7	41.5	37.3	32.0	28.0
1.002	150.0	127.0	105.0	85.0	70.0	63.0	55.0	48.0	43.8	36.4	32.0
1.001	225.0	180.0	140.0	110.0	89.0	75.0	65.0	56.0	50.0	41.8	36.4
1.000	500.0	270.0	220.0	155.0	118.0	98.0	80.0	68.0	59.0	49.0	42.4

The constants for each temperature are calculated as follows: density of test fluid $= K/t + d$; where K and d are constants for the temperature at which the determination was done and t is the falling time. The constants, K and d, for each temperature are plotted against the temperature and from these charts, tables such as Table I are made.

RESULTS

Using the figures in Table I, we have found the mean deviation of various test fluids to be less than $\pm 1 \times 10^{-4}$ and the maximum deviation has been $\pm 2 \times 10^{-4}$ when compared to values obtained with pyknometry. When accuracy to 10^{-3} is sufficient, a table such as Table II may be prepared. This eliminates the calculations altogether.

SUMMARY

The density of body fluids of low density range such as spinal fluid, transudates, some exudates, urine, and ascitic fluid may be determined rapidly and simply with a maximum deviation of $\pm 2 \times 10^{-4}$. The quantity of fluid required is less than 0.03 cu. mm. The method, therefore, is of particular value when accuracy is required and only small volumes are available. It has been found especially valuable in pediatrics.

The use of an oil medium which has a high boiling point permits a single standardization and therefore eliminates the necessity for standards and nomograms. The range of density covered is from 1.000 to 1.023. The density of body fluids of higher density may be determined by modifications of the method which have been described previously.[1, 2]

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CARDIOLIPIN ANTIGEN IN THE KLINE TEST FOR SYPHILIS

IV. Effect of Rate of Rotation of Antigen-Serum Mixture

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BASIC to development of a standardized serodiagnostic test for syphilis is precise specification of all important variables. The rate of rotation of the antigen-serum mixture in the Kline test has been observed to be such a factor. Kline[1] specified "hand rotation" on a flat surface in a three-fourths inch circle at 180 times per minute. Alternatively, the use of the Boerner type rotator set at 180 r.p.m. has been suggested.[2] Kline stated, "Machine rotation must be set by comparison of results with those of standard hand rotation."[1] No data, however, have been reported on the range of readings obtained by different methods of rotation. In the present study, three different types of mechanical rotators operating at different rotations per minute were compared. Significant differences in results of the Kline test were observed.

PROCEDURE

Two hundred seven sera were examined by the Kline test for syphilis employing three different mechanical slide rotators.[3] The speeds of rotation of the machines without load were: (1) 57 r.p.m., (2) 185 r.p.m., (3) 306 r.p.m.* With a uniform load of five 3-inch by 2½-inch agglutination slides the speeds decreased to 56, 182, and 301 r.p.m., respectively. A commercial preparation of standardized cardiolipin-lecithin solution (La Motte) was employed.

RESULTS

Uniformly negative results were obtained on 102 sera with the three rotators. The remaining 105 sera exhibited some reactivity. Exclusive of four sera, the tests rotated at the slowest speed appeared in largest aggregates. In seventy-eight sera, the rotation at 182 r.p.m. gave higher readings than did rotation at 301 r.p.m. Similarly, in eighty-two sera, rotation at 56 r.p.m. gave higher readings than did rotation at 182 r.p.m. When the results of three speeds of rotation were compared, those obtained at 56 r.p.m. showed the highest reading on eighty-two sera. In twenty-eight instances (27 per cent) of the 105 reactive sera, the higher readings altered the results so that "negative" sera became

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* 57 r.p.m.—Three Dimensional Rotator, A. S. Aloe Company, St. Louis, Mo.
185 r.p.m.—Yankee Rotator, Clay-Adams Co., New York, N. Y.
306 r.p.m.—Fisher-Kline Rotator, Fisher Scientific Company, Pittsburgh, Pa.

"weakly positive," or "weakly positive" sera became "positive." The discrepancy is somewhat minimized, however, by the fact that in sixteen of the twenty-eight sera the difference in readings occurred between those designated "slightly rough" (∓) and "weakly positive" (±). In the remaining twelve sera (11 per cent), however, significant differences were noted between "weakly positive" readings at 182 r.p.m. and "positive" readings at 56 r.p.m.

TABLE I. RESULTS OF THE KLINE TEST FOR SYPHILIS OBTAINED ON 105 REACTIVE SERA EMPLOYING THREE MECHANICAL ROTATORS SET AT DIFFERENT SPEEDS OF ROTATION

SPEED OF ROTATION	(1) 56 R.P.M.	(2) 182 R.P.M.	(3) 301 R.P.M.
	Number of sera in which one speed of rotation gave higher readings than another.		
Comparing 2 with 3		78	1
Comparing 1 with 2	82	3	
Comparing 1 with 3	94		1
Comparing 1, 2, and 3	82	3	0
	Number of sera in which one speed of rotation altered the reading to a degree which necessitated a change to a higher report.*		
Comparing 2 with 3		18	0
Comparing 1 with 2	28	0	
Comparing 1 with 3	46		0
Comparing 1, 2, and 3	28	0	0

*Readings scored as follows: negative = 0, ∓ (slight roughness); weakly positive (small clumps) ±, 1+; positive (medium and large clumps) 2+ to 4+.

DISCUSSION

Significant differences in results were obtained in the Kline test using three mechanical rotators operating at different speeds of rotation. Thus, it is evident that hand rotation would be unsuitable as a standard reference procedure because of inherent variability.

The primary determinant of antigen sensitivity is the lecithin-cardiolipin ratio. Obviously a comparative evaluation of various lecithin-cardiolipin ratios is negated by failure to employ a uniform speed of rotation. Kline and Suessenguth[4] reported the optimal lecithin-cardiolipin ratio for the Kline test to vary between 9:1 and 11:1. Klein and Leiby,[5] using a 56 r.p.m. machine, selected for routine use an 8:1 ratio in order to reduce the number of false-positive reactions. In a group of 168 nonsyphilitic sera selected on the basis of a false positive flocculation test, the antigen with a ratio of 8:1 eliminated 27 per cent of sixty-nine false positive or doubtful reactions obtained with an antigen having a ratio of 10:1. The 8:1 ratio represented a compromise selected primarily on the basis of high specificity, but which also approximated maximal sensitivity.

Possibly the results showing the lower specificity obtained by Klein, Konwaler, Sears, Berke, and Leiby[6] was due to the use of the more sensitive rotation rate of 56 r.p.m. Further data are required to determine whether an antigen with a lecithin-cardiolipin ratio of 8:1 will prove optimal when the tests are rotated at 180 r.p.m.

CONCLUSIONS

Significant differences in results were obtained with the Kline cardiolipin slide test for syphilis when rotated on each of three mechanical rotators operating at 56, 182, and 301 r.p.m., respectively.

Sensitivity of the test varied inversely with the rate of rotation.

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INACCURACIES IN THE DISC METHOD FOR DETERMINING BACTERIAL SENSITIVITY TO AUREOMYCIN DISCOVERED BY COMPARISON WITH THE TUBE-DILUTION METHOD

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LABORATORY tests for sensitivity to antibiotics are of value in selecting therapy for infections due to those bacteria which vary widely in sensitivity from strain to strain. Staphylococci, enterococci, members of the coli-aerogenes group, and proteus organisms are important examples of bacteria which display such variability.[1] In determining sensitivity of bacteria to antibiotics, many laboratories are using the disc method in preference to the tube-dilution method. The disc method has become popular because its technique is simple, the results are obtained rapidly, and the discs are distributed commercially. The test is based on the observation that after incubation at 37° C. a zone of bacterial inhibition surrounds a disc resting on the surface of an agar plate heavily seeded with an organism sensitive to the antibiotic contained in the disc. The degree of sensitivity is believed to be represented by the width of the zone of inhibition. The results have been expressed quantitatively by correlating the size of the clear zones with the values for sensitivity determined by the standard tube-dilution test or other techniques which employ known concentrations of antibiotic.[2-4] The present report compares the results obtained when commercially prepared discs were tested in parallel with the tube-dilution method against 74 strains of bacteria immediately after isolation from human infections. We selected a product known as "Dia-Disc"* for trial because these are the only antibiotic-discs whose experimental development had been adequately described in medical literature before commercial distribution was attempted.[2]

METHOD

Aureomycin, terramycin, streptomycin, chloramphenicol, and penicillin were the antibiotics examined in parallel tests with the two methods. The technique used for the Dia-discs was essentially that recommended by the authors. In addition, trypticase soy agar containing 10 per cent rabbit blood was compared simultaneously with trypticase soy agar containing no blood. Interpretation of results was based on the chart provided with the discs. In the tube method, serial twofold dilutions were made of all antibiotics in tryptose phosphate broth. The highest concentration per milliliter was 25.6 micrograms, and the least concentration was 0.1 microgram. The same numerical values were used for dilutions of penicillin in tryptose phosphate broth, except that the concentrations were expressed in units instead of micrograms. Fresh solutions of aureomycin were prepared daily and of terramycin every other day. Concentrated solutions of the other antibiotics were prepared weekly and stored at 4° C.

The broth containing the antibiotic and the agar plates to be used with the discs were inoculated in rapid succession with bacteria transferred from the same twenty-four-hour

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TABLE I. SENSITIVITY IN MICROGRAMS PER MILLILITER OF ANTIBIOTIC FOR VARIOUS STRAINS OF BACTERIA AS DETERMINED SIMULTANEOUSLY IN BROTH AND BY DISC METHOD

STRAIN	AUREOMYCIN		CHLORAMPHENICOL		TERRAMYCIN		STREPTOMYCIN	
	TUBE	DISC	TUBE	DISC	TUBE	DISC	TUBE	DISC
				A. aerogenes				
CP	1.6	4.9*	6.4	0.6-9.3‡	1.6	0.25-2.0	12.8	0.8-6.7§
BA	12.8	N.I.†	25.6	N.I.	6.4	4.1*	6.4	0.8-6.7
SC	*3.2*¶	N.I.	12.8	N.I.	6.4	4.1*	6.4	0.8-6.7
HQ	*0.8*	N.I.	6.4	0.6-9.3	3.2	1.0-4.1	6.4	0.8-6.7
TL	*1.6*	N.I.	6.4	0.6-9.3	3.2	1.0-4.1	1.6	0.8-6.7
RT	*0.4*	N.I.	3.2	0.6-9.3	0.8	1.0-4.1	6.4	0.8-6.7
JB	*0.2*	N.I.	3.2	0.6-9.3	0.8	1.0-4.1	6.4	0.8-6.7
HT	*0.4*	N.I.	6.4	9.3*	1.6	1.0-4.1	N.I.	N.I.
RR	*3.2*	N.I.	6.4	0.6-9.3	3.2	1.0-4.1	N.I.	N.I.
RP	*6.4*	N.I.	*25.6*	0.6-9.3	3.2	1.0-4.1	N.I.	N.I.
DM	*0.8*	N.I.	*3.2*	N.I.	0.8	1.0-4.1	*25.6*	0.8-6.7
FG	*0.4*	N.I.	3.2	0.6-9.3	1.6	1.0-4.1	25.6	N.I.
TK	*0.8*	4.9*	3.2	0.6-9.3	1.6	1.0-4.1	N.I.	N.I.
				B. proteus				
EM	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.	*N.I.*	0.8-6.7
BG	0.2	0.6-4.9	25.6	N.I.	0.8	1.0-4.1	*N.I.*	0.8-6.7
RT	*3.2*	N.I.	N.I.	N.I.	N.I.	N.I.	*N.I.*	6.7*
AB	12.8	N.I.	12.8	N.I.	N.I.	N.I.	*25.6*	0.8-6.7
MS	25.6	N.I.	6.4	0.6-9.3	12.8	N.I.	6.4	0.8-6.7
HD	*0.8*	N.I.	3.2	0.6-9.3	6.4	N.I.	6.4	0.8-6.7
EB	25.6	N.I.	6.4	0.6-9.3	N.I.	N.I.	*N.I.*	0.8-6.7
SB	N.I.	N.I.	12.8	0.6-9.3	N.I.	N.I.	*N.I.*	0.8-6.7
MD	12.8	N.I.	6.4	9.3*	12.8	N.I.	12.8	0.8-6.7
				Ps. aeruginosa				
LC	*6.4*	N.I.	N.I.	N.I.	12.8	N.I.	*N.I.*	0.8-6.7
RT	N.I.	N.I.	25.6	9.3*	N.I.	N.I.	N.I.	N.I.
RH	25.6	4.9*	*3.2*	N.I.				
RT	N.I.	N.I.	25.6	N.I.	0.8	1.0-4.1	6.4	0.8-6.7
AE	12.8	N.I.	25.6	N.I.	3.2	4.1*	*25.6*	1.0-4.1
				E. Coli				
BG	*0.8*	4.9*	3.2	0.6-9.3	0.4	1.0-4.1	N.I.	N.I.
OW	*0.8*	N.I.	6.4	0.6-9.3	1.6	1.0-4.1	*N.I.*	*0.8
KB	*0.4*	4.9*	3.2	0.6-9.3	1.6	1.0-4.1	12.8	0.8-6.7
RB	*0.4*	N.I.	6.4	0.6-9.3	0.8	1.0-4.1	25.6	0.8-6.7
DA	*1.6*	N.I.	3.2	0.6-9.3	6.4	4.1*	25.6	0.8-6.7
SC	*0.8*	N.I.	12.8	N.I.	1.6	1.0-4.1	12.8	0.8-6.7
SC	*0.4*	4.9*	1.6	0.6-9.3	0.8	1.0-4.1	N.I.	N.I.
MD	3.2	4.9*	6.4	0.6-9.3	3.2	0.25-2.0	*12.8*	*0.8
AC	*1.6*	N.I.	6.4	9.3*	1.6	1.0-4.1	6.4	N.I.

broth culture. In this way, the two types of test were carried out simultaneously using the same seed culture for each. In the tube test, 0.1 ml. of culture diluted 10^{-1} was added to 4.0 ml. of each dilution of antibiotic in broth. The bacteria and antibiotic were mixed thoroughly and incubated at 37° C. The tubes were examined grossly for turbidity at six hours and twenty hours. The sensitivity was expressed as the smallest concentration of antibiotic in which visible bacterial growth was absent. Sensitivities were recorded on the basis of readings at six hours if heavy growth had occurred in the broth containing no antibiotic at that time. Otherwise, the results are based on the twenty-hour readings.

RESULTS

In most of the tests for sensitivity to aureomycin there were marked discrepancies between the results obtained by the two methods as recorded in

TABLE I—CONT'D

STRAIN	AUREOMYCIN		CHLORAMPHENICOL		TERRAMYCIN		PENICILLIN	
	TUBE	DISC	TUBE	DISC	TUBE	DISC	TUBE	DISC
			M. pyogenes (Staph. aureus coagulase positive)					
CF	0.2	0.6-4.9	6.4	0.6-9.3	0.2	0.25-2.0	N.I.	N.I.
LD	0.4	0.6-4.9	12.8	0.6-9.3	0.2	0.25-2.0	N.I.	N.I.
RW	*0.1*	0.6-4.9	12.8	0.6-9.3	*0.1	0.25-2.0	*0.1	1.8*
BA	*0.1*	0.6-4.9	*12.8*	*0.6	0.2	0.25-2.0	0.2	*1.8
HJ	12.8	N.I.	12.8	0.6-9.3	N.I.	N.I.	N.I.	N.I.
BK	*0.1*	0.6-4.9	1.6	0.6-9.3	*0.1	0.25-1.0	*0.1	*1.8
BM	*0.1*	0.6-4.9	3.2	0.8-6.7	*0.1	0.25-1.0	0.2	*1.8
DB	*0.4*	N.I.	3.2	0.6-9.3	1.6	0.25-2.0	N.I.	N.I.
IF	0.8	0.6-4.9	6.4	0.6-4.9	0.8	0.25-2.0	*N.I.*	1.8-7.3
RH	12.8	N.I.	25.6	N.I.	*N.I.*	0.25-1.0	N.I.	N.I.
EB	*0.1*	0.6-4.9	3.2	0.6-9.3	*0.1	0.25-1.0	N.I.	N.I.
CP	*0.1*	0.6-4.9	6.4	9.3*	*0.1	1.0-4.1	N.I.	N.I.
NS	*0.1*	0.6-4.9	6.4	0.6-9.3	0.4	1.0-4.1	6.4	3.6*
WG	3.2	N.I.	3.2	0.6-9.3	25.6	N.I.	N.I.	N.I.
HG	*0.1*	0.6-4.9	3.2	0.6-9.3	*0.1	0.25-1.0	*0.1	*0.9
HG	*0.1*	0.6-4.9	6.4	0.6-9.3	0.2	0.25-1.0	*0.1	*0.9
DS	*0.1*	0.6-4.9	12.8	0.6-9.3	0.4	0.25-1.0	N.I.	N.I.
ES	*0.1	*0.6	6.4	0.6-9.3	0.2	0.25-1.0	*0.1	*0.9
WG	6.4	4.9*	12.8	9.3*	25.6	N.I.	1.6	1.8-7.3
EG	*0.1*	4.9*	1.6	0.6-9.3	*0.1	0.25-1.0	0.4*	1.8
HT	0.2	0.6-4.9	6.4	0.6-9.3	0.4	0.25-1.0	N.I.	N.I.
DI	*3.2*	N.I.	1.6	0.6	25.6	N.I.	6.4	1.8-7.3
GK	*0.1*	0.6-4.9	3.2	0.6-9.3	0.2	0.25-1.0	0.2	0.25-1.0
MC	25.6	N.I.	6.4	0.6-9.3	N.I.	N.I.	N.I.	N.I.
EH	*0.1*	0.6-4.9	6.4	0.6-9.3	0.2	0.25-1.0	*3.2*	N.I.
			Enterococcus					
GA	N.I.	N.I.	N.I.	0.6-9.3	N.I.	N.I.	12.8	7.3
HR	25.6	N.I.	6.4	0.6-9.3	N.I.	N.I.	12.8	1.8-7.3
TK	*0.8*	4.9*	3.2	9.3*	1.6	0.25-1.0	12.8	1.8-7.3
RB	*0.1*	4.9*	1.6	9.3*	0.2	0.25-1.0	3.2	1.8-7.3
RH	12.8	N.I.	6.4	N.I.	N.I.	N.I.		
AB	25.6	N.I.	6.4	9.3*	N.I.	N.I.	3.2	1.8-7.3
IM	*0.1*	0.6-4.9	3.2	0.6-9.3	0.2	0.25-1.0	3.2	1.0-4.1
SC	25.6	N.I.	*3.2*	N.I.	N.I.	N.I.	6.4	1.8-7.3
FB	*0.8*	N.I.	6.4	0.6-9.3	3.2	4.1*	6.4	1.8-7.3
JA	0.2	0.6-4.9	0.4	0.6-9.3	3.2	0.25-2.0	6.4	1.8-7.3
JA	0.2	0.6-4.9	0.4	0.6-9.3	3.2	0.25-2.0	6.4	1.8-7.3
TF	*0.1*	N.I.	3.2	0.6-9.3	0.2	1.0-4.1	6.4	1.8-7.3
HR	25.6	N.I.	3.2	0.6-9.3	N.I.	4.1*	N.I.	1.8-7.3

*Italicized figures indicate marked discrepancy between tube method and disc method. Asterisk preceding a figure—less than. Asterisk following a figure—more than.

†N.I.—No inhibition.

‡When two figures are listed, they represent the range of sensitivity in micrograms per milliliter.

Table I. Most strains of staphylococcus, *A. aerogenes*, and *E. coli* were far more sensitive to aureomycin by the tube-method than they appeared to be by the disc method. Although not inhibited at all by the aureomycin discs, most strains of *A. aerogenes* and *E. coli* were more sensitive to aureomycin by the tube test than to any other antibiotic. Thirteen strains of staphylococcus were inhibited by 0.1 microgram or less of aureomycin per milliliter of broth, but fell into the relatively resistant range of 0.6 to 4.9 micrograms of aureomycin per milliliter when the discs were used for determination. Successful treatment of clinical infections due to strains sensitive to aureomycin *in vitro* bore out the reliability of the tube method.

Marked discrepancies were also noted between the results obtained by the two methods for six of the nine strains of *B. proteus* tested for sensitivity to streptomycin. Five of the strains were not inhibited by streptomycin in broth, and the sixth only by 25.6 micrograms per milliliter. Yet all six strains were sensitive to 0.8 to 6.7 micrograms of streptomycin by the disc technique.

There was good correlation between the results obtained by the two methods for chloramphenicol, terramycin, and penicillin against all strains, and for streptomycin against *E. coli* and *A. aerogenes*.

COMMENT

The tube-dilution method has proved to be a useful guide to antimicrobial therapy in this hospital and elsewhere. Infections treated successfully by a given antibiotic are usually caused by bacterial strains which can be inhibited by low concentrations of the same antibiotic in broth. The disc method tested in this study appears to provide results comparable to those of the tube-dilution method for chloramphenicol, terramycin, penicillin, and usually streptomycin. It cannot be relied upon, however, for determining sensitivity to aureomycin. Aureomycin would almost never have been used clinically in this study if selection of antibiotics had been based on the disc method. Yet aureomycin is often used successfully in the treatment of infections caused by members of each of the three species (*A. aerogenes, E coli,* staphylococcus) whose inhibition by the aureomycin discs was consistently absent or weaker than that expected from the tube-dilution test.

In the experimental development of an aureomycin-disc, at least two precautions must be taken to guard against the instability of aureomycin in solution when comparisons are made between the zones of inhibition on agar and the minimum inhibiting concentrations of aureomycin in broth. First, fresh solutions of aureomycin should be prepared daily because even concentrated solutions show marked deterioration after storage in the refrigerator for forty-eight hours.[6] Dilute solutions of aureomycin in broth at 37° C. deteriorate still more rapidly. A second important precaution, therefore, is to shorten the lag phase of bacterial growth as much as possible during the performance of the sensitivity test in broth. Jackson and Finland[5] have pointed out that the lag phase can be shortened to less than six hours in broth by use of a 10^{-2} dilution of the original seed culture. We have used slightly heavier inoculums in the present study for the same purpose, and have found that most sensitivity tests were easily read in six hours, a period during which the deterioration of aureomycin can be kept to a minimum. This technique also has the advantage of providing huge initial bacterial populations (approximately 10^{12} organisms) so that resistant variants are more likely to be present and manifest during the sensitivity test than in the usual procedure wherein the small initial inoculum is used. The use of larger inoculums may thus explain the observed resistance of *B. proteus* to streptomycin in the tube test when the same strains of *B. proteus* appeared sensitive to streptomycin in the disc test. An occasional colony resistant to streptomycin sometimes appears in the clear zone of inhibition surrounding the disc.[4]

On agar, the occasional colony in the clear zone might be overlooked, but in broth the resistant variants have a better chance to manifest themselves by producing a diffusely turbid growth.

Because of the possibility that rabbit blood might contain inhibitors for aureomycin, tests were performed on trypticase soy agar with blood and on trypticase soy agar in the absence of blood. Omission of blood did not influence the activity of the aureomycin-disc. Neter, Murdock, and Kunz[7] found that the degree of inhibition produced by any of six antibiotic discs tested was not materially affected by the addition of 5 per cent human blood. We have concluded that blood need not be added to the agar when sensitivities are done by the disc method.

It was interesting to observe, incidentally, that of 25 strains of staphylococcus tested by the tube-dilution test, 16 strains must be considered resistant to penicillin, but only 6 resistant to terramycin and 6 resistant to aureomycin. Staphylococci were seldom as sensitive to chloramphenicol as they were to either aureomycin or terramycin. The disc method failed to disclose that staphylococci were more sensitive to aureomycin than to chloramphenicol.

SUMMARY AND CONCLUSIONS

There was good agreement between the results obtained upon determining sensitivities of 74 bacterial strains to chloramphenicol, terramycin, and penicillin with a tube-dilution test and with a test employing commercially prepared discs containing antibiotics (Dia-disc).

The disc method examined in this study is not satisfactory for determining bacterial sensitivity to aureomycin. Most strains of staphylococcus, *A. aerogenes*, and *E. coli* were far more sensitive to aureomycin by the tube method than they appeared to be by the disc method.

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ADDITIONAL USES FOR POLYVINYL ALCOHOL IN PARASITOLOGIC DIAGNOSIS

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POLYVINYL alcohol* (PVA), a mounting medium used extensively by mycologists,[1] has also been incorporated into a fixative for intestinal protozoa.[2-4] The addition of the fixative to fresh stool specimens preserves both trophozoites and cysts indefinitely. Organisms in stools preserved in this manner take an excellent iron hematoxylin stain. It has been stated[4] that the method is generally unsuitable for protozoan cysts, and that cysts in slides prepared from fecal material thus preserved frequently show distortion or collapse. In our experience, however, this is true only if the slides are allowed to dry after the smears are made.

In an attempt to develop a method whereby concentrates of protozoan cysts could successfully be stained with iron hematoxylin, various strengths of PVA were added to the concentrate to fix the material to the slide for staining. The method outlined below gave excellent results. Slides thus prepared will contain a relatively large number of organisms which possess the added advantage of exhibiting the cytologic detail of a hematoxylin stain. We consider the method to be useful as a diagnostic procedure when only small numbers of organisms are encountered; it also lends itself to the preparation of teaching slides.

The technique is as follows: (a) Concentrate a portion of feces containing cysts by the Faust zinc-sulfate technique[5]. (b) Place two loopfuls of the concentrate on a clean glass slide, and add three small drops of a 2.5 per cent aqueous solution of PVA. Mix well with a dissecting needle. (c) Spread the suspension in a thin even film approximately the size of a standard square coverslip (22 by 22 mm). (d) Immediately immerse the slide for a minimum of twenty minutes in a saturated solution of mureuric chloride in 70 per cent ethyl alcohol to which just prior to use 5 per cent acetic acid is added. A saturated solution of mercuric chloride in 70 per cent alcohol is used instead of the customary Schaudinn's solution because PVA is soluble in the latter fixative. (e) Stain with Heidenhain's iron hematoxylin. Do not allow slides to dry at any stage in the procedure.

Polyvinyl alcohol may also be used in the preparation of semipermanent mounts of helminth ova. Ova, obtained by concentration, are placed on a slide, mixed with a few drops of PVA fixative, and coverslipped. The preparations are satisfactory for a period of several weeks, and exhibit little or no distortion.

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THE USE OF THE VOLUMETRIC RESPIROMETER IN THE DETERMINATION OF PLASMA CARBON DIOXIDE

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INTRODUCTION

SCHOLANDER and associates[1] have shown that, using the volumetric respirometers, carbonate solutions containing 100 to 300 volumes per cent of CO_2 could be determined. However, levels of CO_2 found in plasma could not be determined with the same degree of precision. In this paper, the possibility of employing the volumetric respirometer for plasma CO_2 analysis was further explored. By using a gas displacement rod of a 2 mm. diameter rather than the 3 mm. rod, the sensitivity was increased to make it possible to determine plasma CO_2. The analyses are carried out on 0.1 ml. samples. Essentially, the method consists of measuring the CO_2 evolved when plasma or CO_2 containing solution is injected into dilute sulfuric acid. In a regular setup with a bath capable of handling eight respirometers, 24 to 32 determinations can be accomplished in an hour.

With slight modification, the method can also be used for determining plasma CO_2 in the presence of ether. This method has been used in the acid-base studies of ether-anesthetized infants.[2] The method for plasma CO_2 determinations in presence of ether by Austin[3] requires 1.0 ml. samples. By using the new respirometers, the determination can be accomplished with 0.2 ml. of plasma.

MATERIALS AND METHODS

Apparatus.—The volumetric respirometers described by Scholander and co-workers[1] were employed. For a full description of the apparatus and the techniques used, the original paper should be consulted.

Procedures.—

A. *Preparation of Small Blood Samples for CO_2 Analyses:* When the supply of blood is ample, any standard technique for separation and storage of blood can be employed. In those instances when blood is limited or in the case of studies on infants a method for preparation of small blood samples for plasma CO_2 determinations is desirable. The following is a description of such a method.

The sample of blood is taken up in a 1 ml. tuberculin syringe containing heparin solution in the dead space, and is then capped. The heparin introduces a dilution error of 1 to 2 per cent which can be empirically determined. The plunger of the syringe is held in place by a spacer cut from a bakelite tubing of appropriate size (Fig. 1, *C*). After the spacer is secured with a rubber band, the syringe is centrifuged (Fig. 1, *B*). The plasma is then transferred into a capillary tubing capped at one end with a rubber injection port (Fig. 1, *D*). This transfer tube contains a drop of mercury which is at first in contact with the injection port.

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As the plasma is introduced into the tubing, the mercury moves out acting as an airtight seal. Plasma samples of 0.1 ml. can then be easily withdrawn using the micro syringe pipette.

B. *Plasma CO_2 Determination:* A quantity of 0.3 ml. of 0.2N sulfuric acid is introduced through a polyethylene tubing into the reaction chamber by means of a tuberculin syringe as shown in Fig. 1, *A*. The plug with the disp'acement rod is then fitted in place and the respirometer is shaken until temperature equilibrium is attained in a water bath at room temperature. A reading, D_0, is made to record the position of the rod at the beginning of the experiment. Then 0.1 ml. of plasma or carbonate solution is delivered through the injection port by means of a micro syringe pipette. The tip of the pipette should first be lowered below the acid surface and the sample delivered slowly. After the delivery, the tip is raised above the acid surface and an equal volume of gas is withdrawn to compensate for the volume

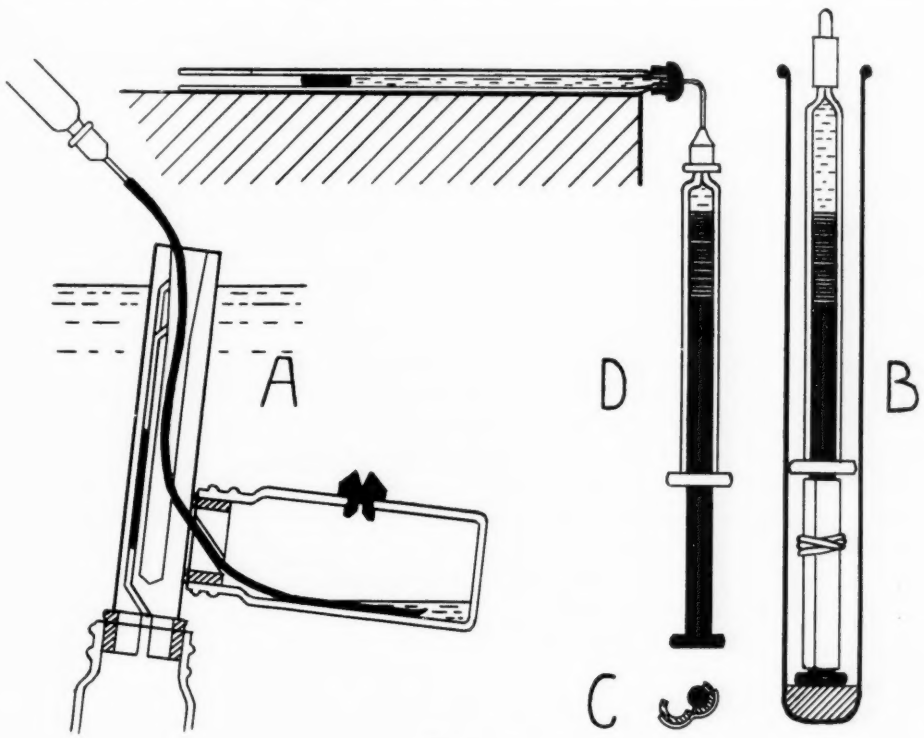

Fig. 1.

of the sample. The pipette is then removed from the chamber and the respirometer is shaken until all the CO_2 is evolved. This is accomplished within one minute. The pressure in the reaction and compensating chambers are made equal by adjusting the gas displacement rod, and the final reading, D_1, is recorded. The temperature of the bath and the barometric pressure are also recorded.

After a determination is completed, the reaction mixture is withdrawn from the chamber by suction through the polyethylene tubing. The reaction chamber is then recharged with sulfuric acid and the respirometer is then ready for another analysis.

C. *Plasma CO_2 Determinations in Presence of Ether:* In this procedure, 0.1 ml. of plasma is injected into a reaction chamber containing 0.3 ml. of 0.2N sulfuric acid. The same amount of plasma is also injected into another chamber containing 0.3 ml. of 0.3N sodium hydroxide. The CO_2 and ether vapor are evolved in the acid chamber, whereas only the ether

vapor is evolved in the alkali. These concentrations of acid and alkali were shown to release equal volumes of ether vapor from a standard aqueous ether solution. Thus, the CO_2 of the plasma can be determined by the difference in the volume change observed in the two chambers.

Calculations: The amount of CO_2 is calculated by multiplying the displacement of the rod by a factor, f.

$$f \times (D_1 - D_0) \text{ in mm.} = \text{mM/L. of } CO_2$$

The calculation for plasma CO_2 in presence of ether is as follows:

$$f \times [(D_1 - D_0)_{acid} - (D_1 - D_0)_{alkali}] = \text{mM/L. of } CO_2$$

The factor, f, depends upon:

1. Rod factor. Each millimeter change in rod excursion is equivalent to a change in gas volume of 3.16 mm.[3]
2. Volume of micro syringe pipette = 98.8 mm.[3]
3. Unextracted CO_2. With the gas phase of 27 ml. and the liquid phase of 0.4 ml., the unextracted CO_2 amounts to 1.1 per cent for temperatures 23 to 27° C.
4. Conversion to S.T.P. B = barometric pressure

   P_w = water vapor tension.
5. Conversion of volume per cent to mM/L. of CO_2.

f is then calculated to be:

$$f = \frac{3.16}{98.8} \times \frac{273}{273 + t° \text{ C.}} \times \frac{B - P_w}{760 \times 22.4} \times 1.011 \times 1{,}000$$

RESULTS

1. CO_2 determinations using the volumetric respirometers were made on standard sodium carbonate solutions ranging in concentrations from 10 mM per liter to 40 mM per liter. These determinations are recorded in Table I. The results, as shown in Table I, reveal that they are in good agreement with the known values. The duplicates agree within ± 0.4 mM per liter. This variation may be expected, as the reading error is from 2 to 3 per cent, which corresponds to 0.2 to 0.4 mM per liter.

2. A comparison was made between the volumetric method for determining plasma CO_2 and the standard Van Slyke-Neill manometric method.[4] The results in Table II indicate that the values obtained by the two methods agree within 2 per cent.

3. Standard sodium carbonate solutions were made containing 1 mg. per milliliter of ether. These solutions were analyzed for CO_2 using the volumetric respirometers. As shown in Table III, the results obtained by this method are within 2 per cent of the known values. The spread of the duplicates is no greater than ± 0.2 mM per liter.

TABLE I. DETERMINATIONS OF CO_2 IN STANDARD SODIUM CARBONATE SOLUTIONS

VALUES KNOWN	10 MM/L.	20 MM/L.	30 MM/L.	40 MM/L.
	9.9	20.1	30.0	39.6
	9.6	19.7	30.6	40.0
Found	10.0	20.1	29.6	39.6
	10.0	20.1	30.1	39.2
	10.3	19.7	29.9	39.2
			30.1	40.0
Average	9.9	19.9	30.1	39.7

4. A comparison was also made between the volumetric method for plasma CO_2 in presence of ether with the Van Slyke method modified by Austin.[3] For this purpose, venous blood was drawn from patients during anesthesia, and the

TABLE II. COMPARISON OF THE VOLUMETRIC PLASMA CO_2 METHOD WITH THE VAN SLYKE-NEILL MANOMETRIC TECHNIQUE

SOURCE OF PLASMA	MANOMETRIC METHOD 1.0 ML. SAMPLE		VOLUMETRIC METHOD 0.1 ML. SAMPLE	
		average		average
			31.1	
Human	30.95		30.9	
	30.86		30.9	
			31.2	
		30.91		31.0
	30.24		30.3	
	30.09		30.1	
Human	30.11		30.4	
			30.1	
		30.11		30.2
	20.20		20.4	
	20.09		19.9	
Horse	20.15		20.4	
			20.3	
		20.11		20.3
			26.8	
			27.0	
Horse	26.83		27.0	
	26.80		26.6	
		26.82		26.9

TABLE III. DETERMINATIONS OF CO_2 IN STANDARD CARBONATE SOLUTIONS WITH 1 MG. PER MILLILITER OF ETHER

Values Known	15 mM/L.	25 mM/L.	35 mM/L.
	14.8	25.1	34.9
Found	15.1	25.3	34.7
	14.7	25.0	34.6
	14.7	24.7	34.6
Average	14.8	25.0	34.7

The values are expressed in mM per liter.

plasma was prepared for analyses by centrifuging under oil. The results of this comparison are shown in Table IV. The values obtained by these two methods agree within 4 per cent except for one sample. With one exception, the spread of the duplicates is no greater than ± 0.3 mM per liter.

SUMMARY

The results reported in this paper indicate that the volumetric respirometers introduced by Scholander and associates[1] can be employed for plasma CO_2 analyses. The volume of sample required is 0.1 ml. It is possible to accomplish 24 to 32 determinations in one hour. The accuracy for a single determination is about 2 per cent which corresponds to 0.3 mM per liter. This method has also been adapted to analyze plasma CO_2 in the presence of ether with about the same degree of accuracy.

TABLE IV. COMPARISON OF THE VOLUMETRIC METHOD WITH THE MANOMETRIC TECHNIQUE FOR THE DETERMINATION OF CO_2 IN PLASMA CONTAINING ETHER

PATIENTS	MANOMETRIC METHOD 1.0 ML. SAMPLE		VOLUMETRIC METHOD 0.2 ML. SAMPLE	
		average		average
	18.9		18.2	
S. W.	18.6		18.4	
			18.2	
		18.8		18.3
	27.6		27.8	
R. H.	27.0		27.8	
		27.3		27.8
	30.6		31.7	
	31.1		32.4	
J. Y.			31.0	
			31.0	
		30.9		31.5
J. Y. I.	29.3		30.8	
	29.9		30.9	
			30.8	
			31.2	
		29.6		30.9
Y. B.	17.0		17.6	
	17.0		17.5	
		17.0		17.6
	27.2		26.6	
B. P.	27.2		26.7	
		27.2		26.7
	26.2		25.7	
R. H.	25.9		25.2	
		26.1		25.5
	16.7		15.7	
R. F.	16.3		16.2	
			15.7	
		16.5		15.9

The values are expressed in mM per liter.

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